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Phospholipid Synthesis During the Cell Division Cycle of *Escherichia coli*

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Stepwise changes in the rate of phosphatidylethanolamine and phospholipid synthesis during the cell division cycle of *Escherichia coli* B/r were observed. The cell ages at the increases were found to be a function of the growth rate. At each growth rate, the increase occurred around the time new rounds of chromosome replication were inaugurated in the cycle.

The manner in which the components of the bacterial envelope (namely, the inner membrane, the cell wall, and, in gram-negative bacteria, the outer membrane) are synthesized during the cell division cycle is still obscure. Zonal growth of peptidoglycan, a component of the cell wall, at a discrete number of sites has been demonstrated by Schwarz and collaborators in gram-negative *Escherichia coli* (7, 15, 23, 25, 26) and by Cole (4), Briles and Tomasz (1), and Burdett and Higgins (2) in gram-positive bacteria. In contrast, nonlocalized synthesis of membrane elements has been suggested, based on the random partition of membrane components at division (8, 27, 28). Stepwise increases in the rates of synthesis of membrane proteins (3, 9, 10, 21, 24) and of phospholipids (9) during the cell division cycle may indicate that the membranes of both gram-negative and gram-positive bacteria are synthesized at a discrete number of cellular sites, similar to the zonal growth of the wall.

Considering the partitioning of the chromosomes into daughters at division (16), it is possible that envelope growth and replication of the chromosomes are coupled. If the processes were coupled, new sites of envelope synthesis might be inaugurated at initiation of new rounds of chromosome replication (22). Envelope synthesis at these sites might continue during chromosome replication and during septum formation. The new sites of envelope synthesis could, therefore, represent potential division sites which would appear in a cell one or more generations prior to division. Alternatively, the rate of envelope synthesis could increase at completion of rounds of chromosome replication as a consequence of initiation of septum-cross wall formation (9, 10, 15). Thus, if envelope synthesis were coupled to the chromosome replication cycle, some or all elements of the envelope might be synthesized at an increased rate either at initiation or at completion of rounds of chromosome replication.

In an effort to elucidate the pattern of envelope synthesis during the bacterial division cycle, I have investigated the synthesis of phosphatidylethanolamine in *E. coli* B/r substrains A, F, and K (14). Phosphatidylethanolamine is found in both the inner and outer membranes of *E. coli* and represents the stable component of the phospholipids in these bacteria (6, 11, 19).

MATERIALS AND METHODS

Bacteria and growth conditions. The organisms used were *E. coli* B/r substrains A, F, and K (14).

The minimal salts medium contained 2 g of NH_4Cl, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 3 g of NaCl, and 0.25 g of $MgSO_4$ in 1 liter of distilled water. Glucose or glycerol at 0.1% final concentration was used as a carbon source. Various amino acids at 50 μg/ml and Casamino Acids at 0.2% (final concentrations) were added as indicated.

Radiochemicals. [2-^{3}H]glycerol (8 Ci/mmol), sodium [1,2-^{14}C]acetate (54 mCi/mmol), [3-^{3}H]serine (46 mCi/mmol), [*U*-^{14}C]leucine (270 mCi/mmol), and [2-^{14}C]thymidine (60 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass.

Phospholipid extraction. Cell samples radioactively labeled with [3-^{3}H]serine were precipitated with ice-cold trichloroacetic acid at 5% final concentration and maintained at 4°C for at least 30 min. Samples were filtered onto 24-mm 4750-H2O filter disks (Arthur Thomas & Co., Philadelphia, Pa.) and washed three times with ice-cold 5% trichloroacetic acid containing nonradioactive serine at 100 μg/ml. A total of 4×10^9 nonradioactive carrier cells, precipitated with trichloroacetic acid at 5% final concentration, were added to the filter containing the ^{3}H-labeled samples. The filters were dried for 40 min at 110°C and extracted in 2 ml of methanol at 55°C for 10 min in tightly stoppered vials. After cooling, chloroform (4 ml) was added. The methanol-chloroform mixture was placed in new vials, each containing a 24-mm filter disk, and evaporated to dryness at room temperature, and the radioactivity on the filters was counted. The incorporation of radioactivity in the methanol-chlo-

roform mixture and in the residue increased linearly within 1 to 7 min after addition of 0.2 μCi of [3-^{3}H]serine per ml to exponential-phase cultures of *E. coli* B/r (Table 1). The radioactivity of the extract was about 25% the radioactivity of the residue. The methanol–chloroform mixture of cells, double-labeled with 1 μCi of [3-^{3}H]serine per ml and 0.1 μCi of [^{14}C]leucine per ml for 5 min, contained less than 1.0% of the ^{14}C radioactivity in the residue, indicating that extraction of protein was negligible.

Samples labeled with [^{14}C]acetate were centrifuged at 5°C, and the cell pellets were washed three times with cold minimal salts medium containing 100 μg of nonradioactive sodium acetate per ml. The number of cells recovered after centrifugation was determined. To each sample 5×10^9 nonradioactive cells were added, and the cell pellets were extracted in 2 ml of methanol at 55°C for 10 min. A 4-ml volume of chloroform was added, and the mixture was blended in a Vortex mixer. The supernatant was separated by centrifugation and transferred into vials, and the radioactivity was counted as indicated above. The incorporation of radioactivity in the extract of exponential-phase *E. coli* B/r cultures is shown in Table 1. Extensive washing of the extracts from synchronous growing *E. coli* B/r cultures with 0.1 M KCl removed about 10% of radioactivity from the lower phase independent of cell age.

The material extractable in the chloroform–methanol mixture of cultures labeled with [2-^{3}H]glycerol has been analyzed by partitioning the components by thin-layer chromatography, as described in detail in a previous communication (29) (Fig. 1). The sum of the radioactivities associated with phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin was equivalent to the radioactivity of the extract prior to partitioning. The partition of the radioactivity in the three components was 68% in phosphatidylethanolamine, 29% in phosphatidylglycerol, and 3% in cardiolipin.

TABLE 1. *Incorporation of [3-^{3}H]serine and [^{14}C]acetate in exponential-phase cultures of E. coli B/r F*[a]

Time (min)	[3-^{3}H]serine		Sodium [^{14}C]acetate
	Methanol–chloroform (cpm/sample)	Residue (cpm/sample)	Methanol–chloroform (cpm/sample)
1	1,001	3,666	284
2	1,799	6,734	459
3	2,738	10,386	645
4	3,551	13,630	792
5	4,753	16,224	1,014
6	—	—	1,196
7	6,446	21,872	1,364
9	7,435	27,858	1,846
11	—	—	2,132

[a] Exponential-phase cultures of *E. coli* B/r F growing in glucose minimal medium (1.5 doublings per h) have been exposed to either 0.2 μCi of [3-^{3}H]serine per ml or 0.08 μCi of sodium [^{14}C]acetate per ml at the time cell concentration was 10^8 cells per ml (time 0). At the times indicated, 5-ml samples were either precipitated in cold trichloroacetic acid at 5% final concentration ([3-^{3}H]serine) or kept in ice till the end of the experiment in the presence of 100 μg of nonradioactive sodium acetate (sodium [^{14}C]acetate). Lipids were extracted into methanol–chloroform as indicated in the text. —, Value not determined.

Incorporation of lipid precursors during the cell cycle. The rate of incorporation of [3-^{3}H]serine during the cell division cycle of *E. coli* was determined with the membrane elution technique (12). Exponential-phase cultures were exposed to 0.2 μCi of [3-^{3}H]serine per ml for 5 min and bound onto a type GS, 142-mm Millipore membrane filter (Millipore Corp., Bedford, Mass.) by suction, and the excess radioactivity was removed by passing 100 ml of medium through the Millipore filter (12). The filter was inverted, and elution was started by pumping medium at 2 to 4 ml/min through the filter. Samples of the effluent were collected continuously during constant time intervals, and the radioactivity in the methanol–chloroform mixture and in the residue was determined as indicated above.

The pattern of incorporation of lipid precursor during the division cycle was also determined by exposing synchronously growing populations to 0.08 μCi of sodium [1,2-^{14}C]acetate per ml for a brief period of time and measuring the radioactive counts in the methanol–chloroform extract. Consecutive 5-ml samples of newborn cells were collected from membrane-bound cultures, grown synchronously for various periods of time at 37°C with shaking (14), and exposed to sodium [^{14}C]acetate for 4 or 6 min at the end of synchronous growth. Incorporation was terminated by adding 100 μg of nonradioactive sodium acetate per ml and cooling to 5°C. The rate of incorporation during synchronous growth was estimated by dividing the radioactive counts in the extract by the number of the cells recovered after centrifugation and multiplying these values by the ratio of the cell concentrations at the end and the start of synchronous growth. The number of cells recovered after centrifugation was 80 to 100% of the cells at the end of synchronous growth. The cell loss was, in general, larger in the samples of younger ages and was negligible in samples of the older ages.

DNA replication pattern. The incorporation of [^{14}C]thymidine during the cell division cycle was determined as described in detail elsewhere (12, 14).

Cell concentration. Cell concentration was determined with a Coulter Counter model F.

Radioactivity. Radioactivity was measured in Spectrafluor (Amersham Corp., Arlington Heights, Ill.) with a Nuclear Chicago scintillation counter.

RESULTS

The pattern of radioactive serine and thymidine incorporation during the division cycle of *E. coli* B/r was determined by briefly exposing exponential-phase cultures growing at a variety of rates to [3-^{3}H]serine and [^{14}C]thymidine, respectively, binding the cells onto a nitrocellulose membrane filter, and measuring the radioactivity of the progeny eluted from the membrane-

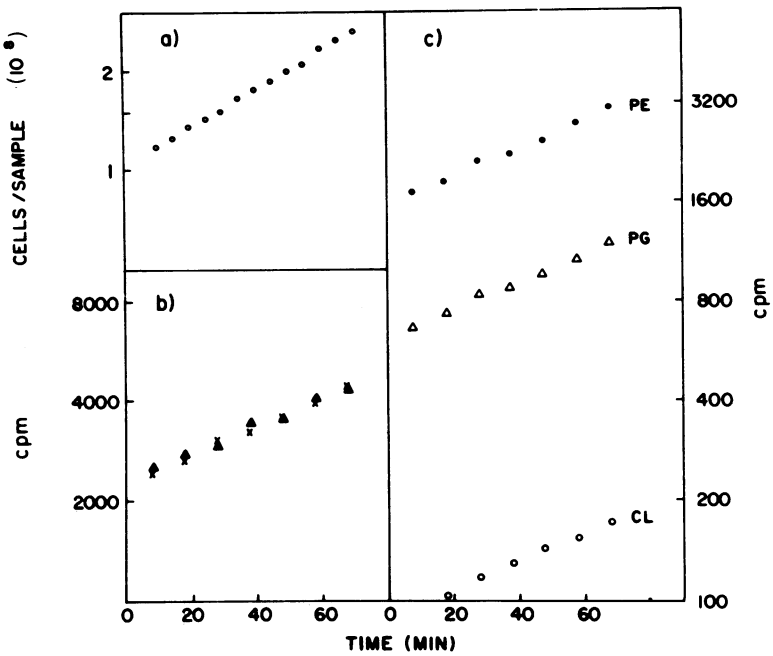

FIG. 1. *Incorporation of [2-^{3}H]glycerol in E. coli B/r A during exponential growth. Samples of 2.5 ml of E. coli B/r A, growing in glucose minimal medium, were exposed to 4 μCi of [2-^{3}H]glycerol per ml for 10 min at various times during growth. Incorporation was stopped by addition of 10 mg of glycerol to each sample and cooling at 5°C. Samples were centrifuged, and the pellets were washed four times with minimal salts medium containing nonradioactive glycerol. A total of 5 × 10^9 nonradioactive cells were added, and each pellet was extracted at 55°C for 10 min with 2 ml of methanol. A 4-ml volume of chloroform was added to the samples, and the mixtures were blended in a Vortex mixer and centrifuged. Samples of 1 ml of the supernatants were transferred into vials, each containing a filter disk, and dried at room temperature, and the radioactivity was counted. Samples of 4 ml were evaporated to dryness at room temperature. The resulting lipid films were dissolved in 0.2 ml of methanol and 0.4 ml of chloroform. Lipids were analyzed by thin-layer chromatography, as indicated in detail elsewhere (29). (a) Cell number per sample. (b) Radioactivity in the methanol-chloroform mixture: (×) sum of the radioactivity counts associated with phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) of 4 ml of extract; (▲) fourfold value of the radioactivity of 1 ml of extract. (c) Radioactivity associated with PE (●), PG (△), and CL (○) of 4 ml of the extract.*

bound population (Fig. 2). The rates of [3-^{3}H]-serine or [^{14}C]thymidine incorporation during the division cycle are visualized by following the curves of radioactivity per effluent cell from right to left in each generation (12). In the culture labeled with [3-^{3}H]serine, the radioactivity in the methanol chloroform extract of the effluent decreased continuously during the first generation of elution, but plateaus in radioactivity were observable during the second and third generation. The stepwise changes in [3-^{3}H]serine radioactivity occurred in correspondence to the stepwise changes in [^{14}C]thymidine incorporation in the parallel culture.

The results of experiments with cultures of *E. coli* B/r F growing at a variety of rates are summarized in Fig. 3. The vertical lines indicate generations of elution as determined by twofold decreases in serine incorporation into the residue (protein fraction), and the arrows indicate generations of elution as determined by the time interval between maxima in the cell concentration in the effluent. The rate of serine incorporation into proteins was continuous during the cell division cycle. The rate of serine incorporation into the lipid fraction increased around the time thymidine incorporation increased in the parallel cultures, i.e., at initiation of new rounds of chromosome replication (12). Similar correspondence between initiation of chromosome replication and the time of the increase in incorporation of [3-^{3}H]serine into the lipid fraction was seen in *E. coli* B/r A and B/r K growing at various rates (data not shown).

Experiments similar to those described in Fig. 2 and 3 were also performed using [2-^{3}H]glycerol incorporation into lipids as the assay procedure. In these experiments, the pattern of radioactivity in the effluent was difficult to interpret, due to turnover of radioactive phospholipids during growth on the membrane filter (29). However, results similar to those in Fig. 2 and 3 were

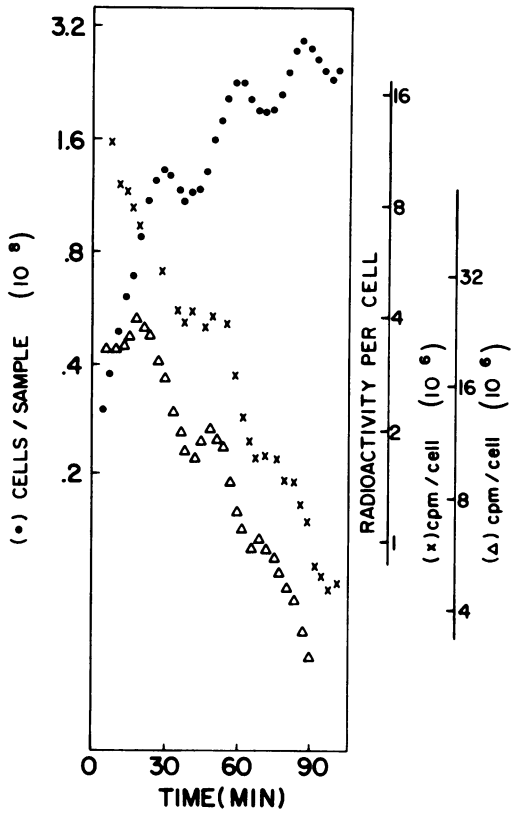

FIG. 2. *Elution profile and radioactivity in the effluent of E. coli B/r K. Exponential-phase E. coli B/r K cultures (100 ml) growing in glucose minimal media supplemented with Casamino Acids and tryptophan were exposed to either 0.2 μCi of [3-^{3}H]serine per ml for 5 min or to 0.01 μCi of [^{14}C]thymidine per ml for 3 min at the time the cell concentration was 10^8 cells per ml. The cultures were bound onto a nitrocellulose Millipore filter and washed with 100 ml of growth medium (12). The filter was inverted, and elution was started with medium of the same composition.* (●) *Number of cells per sample and* (×) *radioactivity in the methanol chloroform extract of the effluent of the culture labeled with [3-^{3}H]serine;* (△) *radioactivity in cold 5% trichloroacetic acid-insoluble material of the effluent of the [^{14}C]thymidine-labeled cultures (12).*

obtained when each sample was allowed to grow for three to four generations after collection. After this period, essentially all of the radioactivity was found to be associated with stable phosphatidylethanolamine.

The rate of incorporation of radioactive acetate and the rate of DNA synthesis during the cell division cycle were determined by briefly exposing synchronously growing populations to either [^{14}C]acetate or [^{14}C]thymidine. The rate of [^{14}C]acetate incorporation as a function of the cell age at the time of the pulse is shown in Fig. 4a, and the rate of DNA synthesis is shown in Fig. 4b. Stepwise increases in the radioactivity were observed. The increases in the rate of [^{14}C]acetate incorporation were at approximately the same stage in the division cycle as, or slightly later than, the increase in [^{14}C]thymidine incorporation. Similar results were also obtained when [2-^{3}H]glycerol was used as a lipid precursor. From experiments such as those in Fig. 2 to 4, the cell ages at the increases in rate in [^{14}C]acetate and [3-^{3}H]serine incorporation into the cell lipids were determined and compared with the ages at initiation of chromosome replication (Fig. 5). The data points generally lie on a straight line with a slope of 1, consistent with a temporal correlation of the two events in the cell cycle.

DISCUSSION

Changes in rate of incorporation of radioactive lipid precursors and of [3-^{3}H]serine into methanol–chloroform extracts of *E. coli* cultures were considered to be a consequence of changes in rate of lipid and of phosphatidylethanolamine synthesis, respectively (11, 17). Equilibration of the exogenous precursors with the lipid precursor pool occurred within 1 min from the exposure of exponential-phase cultures of *E. coli* B/r to [^{14}C]acetate, [3-^{3}H]serine (Table 1), and [2-^{3}H]-glycerol (data not shown). Thereafter, the incorporation increased linearly with time. Incorporation of radioactivity terminated immediately upon addition of nonradioactive precursors to the cell cultures (18, 29). Leduc and Schaechter (18) have previously shown that in *E. coli* the size of the lipid precursor pool is extremely small.

In *E. coli* B/r the rate of [3-^{3}H]serine incorporation, and thus of phosphatidylethanolamine synthesis, increased in a stepwise fashion during the cell division cycle, about coincident with initiation of rounds of chromosome replication. Since the composition of newly synthesized phospholipids is independent of the age of the cells (29), the observed increase in rate of synthesis of phosphatidylethanolamine must be accompanied by an increase in the rate of synthesis of the two other phospholipid components, phosphatidylglycerol and cardiolipin. The increase in the rate of [^{14}C]acetate incorporation in the phospholipid extracts, approximately coincident with the initiation of rounds of chromosome replication, is consistent with these findings.

The increase in phospholipid synthesis could be a consequence of the activation of new sites of envelope growth, and if this were the case, it would be accompanied by a simultaneous in-

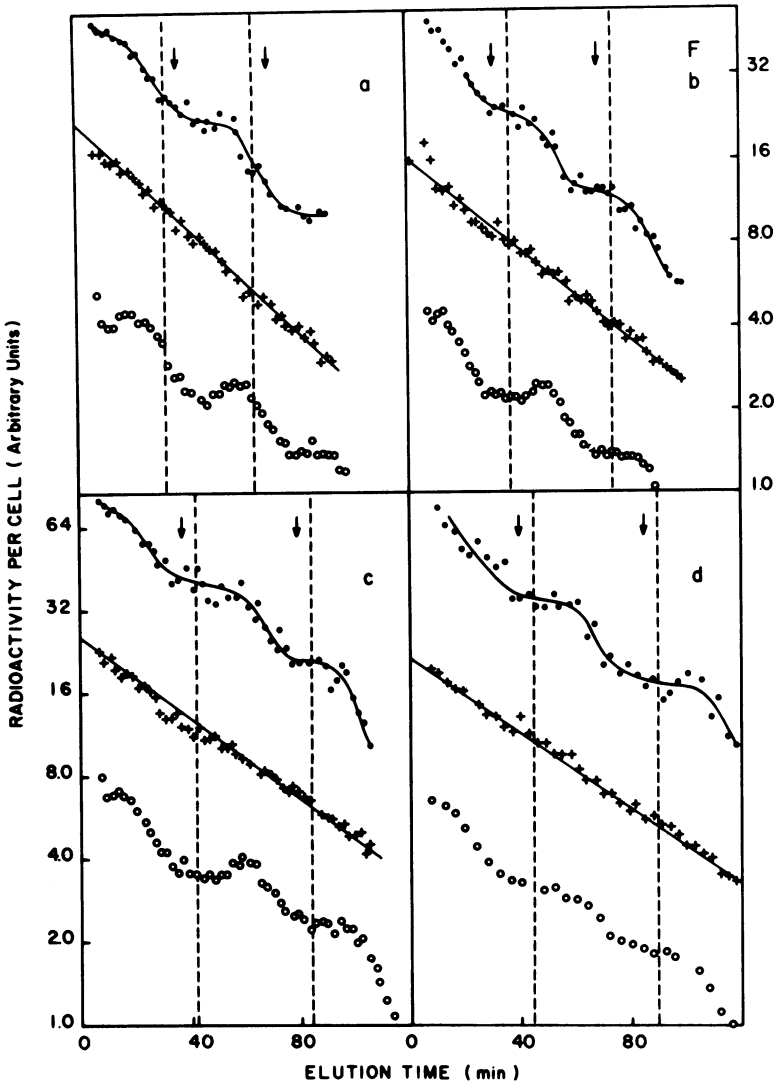

FIG. 3. *Pattern of [3-^{3}H]serine and [^{14}C]thymidine incorporation in the effluent of E. coli B/r F. Exponential-phase cultures of E. coli B/r F (100 ml) containing 10^8 cells per ml were pulse-labeled for 5 min with 0.2 μCi of [3-^{3}H]serine per ml or 0.01 μCi of [^{14}C]thymidine per ml and bound onto the bottom surface of a nitrocellulose membrane filter, and the radioactivity in cells in the effluent from the membrane was determined.* (●) *^{3}H radioactivity in the methanol-chloroform extract of the effluent cells (phosphatidylethanolamine).* (×) *^{3}H radioactivity in the residue after extraction (protein).* (○) *^{14}C radioactivity in a separate culture. The vertical interrupted lines indicate generations of elution as measured by the time required for the radioactivity per cell in the protein fraction to decrease twofold. The arrows indicate generations of elution as measured by the interval between maxima in the cell concentration in the effluent. The growth rates, media compositions, and radioactivity per 10^6 cells at the start of elution in phosphatidylethanolamine, protein, and DNA, respectively, were as follows. (a) 1.88 doublings per h; glucose supplemented with 18 amino acids (histidine, methionine, arginine, tryptophan, phenylalanine, leucine, proline, threonine, isoleucine, alanine, glycine, tyrosine, lysine, cysteine, glutamic acid, glutamine, aspartic acid, and asparagine); 9, 30, and 42 cpm. (b) 1.62 doublings per h; glucose supplemented with 12 amino acids (histidine, methionine, arginine, tryptophan, phenylalanine, leucine, proline, threonine, isoleucine, alanine, glycine, and tyrosine); 6.5, 25, and 35 cpm. (c) 1.42 doublings per h; glucose supplemented with five amino acids (histidine, methionine, arginine, tryptophan, and phenylalanine); 12, 36, and 39 cpm. (d) 1.33 doublings per h; glucose supplemented with histidine; 10, 39, and 29 cpm.*

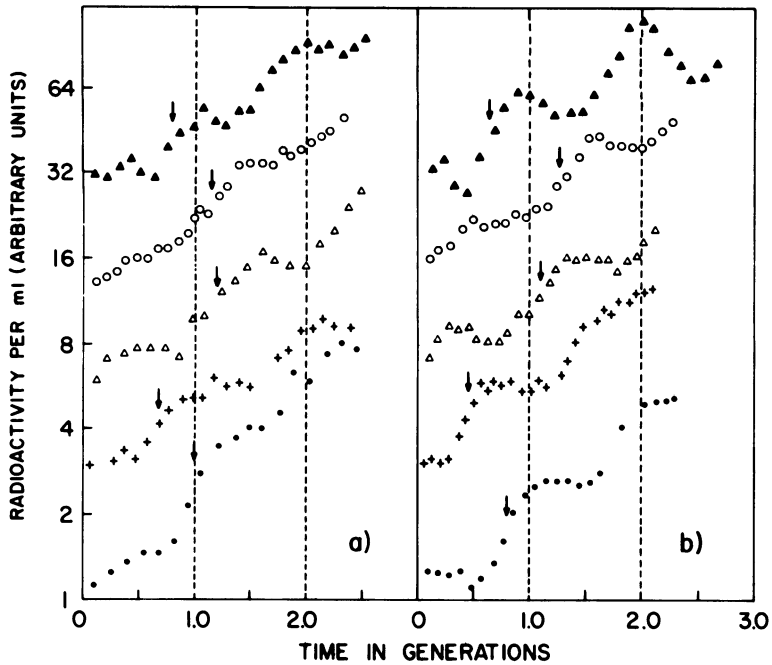

FIG. 4. *Rate of incorporation of (a) [^{14}C]acetate and (b) [^{14}C]thymidine in synchronous growing populations of E. coli B/r A. (a) Cultures of 5 ml of E. coli B/r A were pulse-labeled with 0.08 μCi of [^{14}C]acetate per ml at a variety of cell ages. The cells were washed and the phospholipid was extracted as indicated in detail in the text. The growth rate, the medium composition, the length of the exposure to [^{14}C]acetate, the number of cells (× 10^8), and the radioactivity in the extract per 10^6 cells in the first sample of each culture were as follows. (●) 1 doubling per h; glycerol; 6 min; 1.4; and 7.8 cpm. (+) 1.2 doublings per h; glucose; 6 min; 2.0; and 1.4 cpm. (△) 1.76 doublings per h; glucose supplemented with methionine, histidine, and arginine; 6 min; 2.0; and 1.6 cpm. (○) 1.9 doublings per h; glucose supplemented with methionine, histidine, arginine, leucine, threonine, and proline; 6 min; 1.1; and 1.0 cpm. (▲) 2.1 doublings per h; glucose supplemented with Casamino Acids and tryptophan; 4 min; 2.0; and 3.1 cpm. (b) Cultures of 5 ml of E. coli B/r A were pulse-labeled with 0.1 μCi of [^{14}C]thymidine per ml at a variety of cell ages. The medium compositions were the same as in (a). The growth rate, the length of the exposure, the number of cells, and the radioactivity per 10^6 cells in 5% cold trichloroacetic acid-insoluble material were as follows: (●) 0.97 doublings per h, 6 min, 1.4, and 20.7 cpm; (+) 1.3 doublings per h, 2 min, 0.7, and 12.1 cpm; (△) 1.86 doublings per h, 4 min, 2.1, and 30.4 cpm; (○) 2.0 doublings per h, 2 min, 2.9, and 22.2 cpm; (▲) 2.2 doublings per h, 4 min, 2.8, and 53.0 cpm. Arrows indicate the midpoints of the increases in rate; the dashed vertical lines are drawn at the midpoints of the increase in cell number.*

crease in the synthesis of other membrane and wall elements. In *E. coli* B/r growing in glucose minimal medium, the synthesis of phospholipid and protein components of the inner and outer membranes, wall-bound murein hydrolases, and murein increase around the time of completion of chromosome replication (9, 10, 15). In *E. coli* K-12 growing in broth medium, murein synthesis increased at cell division (19). Since rounds of chromosome replication might initiate at termination of the previous round in cells growing in glucose minimal medium and at division in cells growing in broth medium (5, 13), my results and those of Hakenbeck and Messer (9, 10), Hoffman et al. (15), and Mathison (19) are consistent with a coordinate increase in rate of synthesis of envelope components at a fixed time in the cycle which could be coincident with initiation of chromosome replication (22). The difference in the numbers of zones of diaminopimelic acid incorporation at intermediate and fast growth rates might also be consistent with activation of sites of murein synthesis at initiation of chromosome replication (23, 26). In contrast, in *E. coli* cells synchronized by amino acid starvation, the amount of newly formed murein increased at division, with the newly synthesized murein attaching to the preexisting cell sacculus after cell division (20). Churchward and Holland (3), Ohki (21), and Sargent (24) reported that the rate of [2-^{3}H]glycerol incorporation increased exponentially during the bacterial division cycle. Stepwise increases in the rate of envelope protein synthesis, which would be consistent with

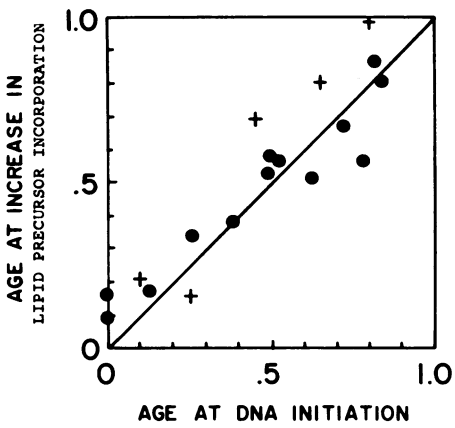

FIG. 5. *Cell age at the increase in rate of incorporation of lipid precursors. The cell ages (●) at the increase in rate of serine incorporation and at initiation of rounds of chromosome replication were determined from experiments of the same type as those in Fig. 2 and 3. The cell ages were measured during the second generation of elution and represent the times in the division cycle corresponding to the midpoints of the increases in radioactivity per cell. (+) Ages determined from Fig. 4, in correspondence to the arrows. The line is drawn with a slope of 1.0.*

zonal growth of the envelope, were observed by these authors. In one case, the increase occurred at about 0.5°C (3); in the others the timing of the increases could not be related to the pattern of chromosome replication either because of the uncertainty due to the synchronization procedure (21) or the lack of information on the chromosome replication pattern (24).

The alternative possibility, that the observed cell cycle-dependent incorporation of radioactive precursors into the cell lipids could be a consequence of fluctuations in either the size of precursor pools or transport activity, cannot be rigorously excluded. However, the pattern of incorporation during the cell cycle was independent of whether serine, glycerol, or acetate was used as radioactive precursor of the cell lipids. Thus, it is unlikely that changes in lipid precursor pool size and transport activity were responsible for the changes in incorporation. Were this proven to be so, then the observed pattern of incorporation would indicate that once in a cycle, at the time rounds of chromosome replication are inaugurated, envelope-related cell properties are specifically affected.

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