Lipid Synthesis in Relation to the Cell Cycle of *Bacillus megaterium* KM and *Escherichia coli*

By M. J. DANIELS

Microbiology Unit, Department of Biochemistry, University of Oxford

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Lipid synthesis during the cell duplication cycle of *Bacillus megaterium* KM and *Escherichia coli* was studied by glycerol incorporation both in synchronized cultures and in unsynchronized exponentially growing populations subsequently fractionated according to size (and age). A large transient increase in the rate of incorporation per unit cell mass was observed around the time of cell division, probably reflecting the synthesis of the division septum.

The importance of the membrane in the organized growth of bacterial cells is now generally recognized. In addition to being the site of diverse enzymic activities and transport systems, the membrane is presumed to effect chromosome segregation and cell division (Ryter, 1968). It is therefore to be expected that membrane synthesis is related in a characteristic fashion to the cycle of cell duplication.

In the present work the incorporation of radioactive glycerol into the lipids of *Bacillus megaterium* KM and *Escherichia coli* was studied in relation to the cell cycle. It seems probable that, in bacteria, glycerol-containing lipids are confined to the cell membrane (O'Leary, 1967), and it is reasonable to suppose that the synthesis of these compounds mirrors to some extent the growth of the membrane. The cell cycle was studied by using synchronized cultures and also by fractionating unsynchronized cultures according to cell size (and therefore age). The latter approach avoids any artifacts that may be introduced by the synchronization process.

B. megaterium KM was chosen because its high sensitivity to lysozyme makes it very suitable for membrane investigations. The membranes of Gram-negative bacteria are as yet poorly characterized (Salton, 1967), but, despite this disadvantage, comparative studies were undertaken with *E. coli* because of the extensive biochemical and genetic information available for this species.

MATERIALS AND METHODS

[2-³H]Glycerol (500 c/mole) and [1-¹⁴C]glycerol (23.6 c/mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Scintillator [2-(4-*tert*.-butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole] was purchased from Ciba Ltd., Duxford, Cambs., and was dissolved in toluene at a concentration of 5g./l. All other reagents were of A.R. grade where possible.

Bacteria: growth and synchronization. A threonine auxotroph of *B. megaterium* strain KM, isolated in this laboratory by Mrs J. Leader, was grown in the salts medium of Davis & Mingioli (1950) supplemented with glucose (0.2%, w/v) and L-threonine (20 µg./ml.), added separately after sterilization. Inocula were prepared by overnight growth of 5 ml. cultures at 37° in test tubes placed in a sloping position; they were then diluted with at least 20 vol. of fresh medium and grown with vigorous aeration at 37°. Threonine deprivation was produced by rapidly filtering an exponentially growing culture under pressure on a Millipore HA membrane filter (diameter 47 mm.). The cells were washed by passing through the filter 40 ml. of warm medium lacking threonine and were then resuspended and incubated with aeration in warm threonine-free medium. After starvation for 60 min., L-threonine was added back at a concentration of 20 µg./ml. to initiate synchronous growth.

E. coli 3/62 (a methionine auxotroph derived in this laboratory from strain 518) was grown in the Davis & Mingioli (1950) salts medium supplemented with glucose (0.2%, w/v) and L-methionine (20 µg./ml.). Synchronization was achieved by the method of Matney & Suit (1966). An overnight culture was centrifuged and the cells were resuspended at a density equivalent to about 0.02 mg. bacterial dry wt./ml. in medium containing L-methionine at a concentration of 1 µg./ml. Growth ceased because of methionine exhaustion when the culture had reached a density equivalent to about 0.15 mg. bacterial dry wt./ml. Incubation was continued with aeration for a further 3 hr. and synchronous growth was then initiated by adding an equal volume of warm fresh medium containing L-methionine at a concentration of 20 µg./ml.

Bacterial growth was measured in a Unicam SP.600 spectrophotometer at 600 nm. The extinction values were converted into bacterial dry wt./ml. by means of calibration curves for the two organisms. Since extinction measurements are more nearly a measure of bacterial mass than of numbers (Koch, 1961) it was assumed that the calibration curves could be applied to synchronous cultures, despite the variation in mean cell size during the cell cycle. This assumption seems justified in view of the apparent exponential increase of bacterial mass/ml. observed during synchronous growth.

Total cell counts were performed with suitable dilutions

of formalin-fixed samples by using a Petroff-Hauser counting chamber and a phase-contrast microscope.

Incorporation of glycerol into lipids. [$2\text{-}^3\text{H}$]Glycerol was added to cultures together with sufficient unlabelled glycerol to ensure continued incorporation throughout the experiment. Samples (usually 1 ml.) were taken and pipetted into 5 ml. of ice-cold trichloroacetic acid (final concentration 5%, w/v) containing unlabelled glycerol (0.5 mg./ml.). The tubes were cooled in ice for 30 min. and the precipitates were collected on Whatman glass-fibre filters (type GF/C, 2.5 cm. diam.), washed three times with 5 ml. of cold 5% (w/v) trichloroacetic acid, and dried in a vacuum oven at 70°. The dried filter was immersed in toluene scintillator solution and the radioactivity determined with a Beckman scintillation counter. Essentially

all the incorporated radioactivity thus measured could be recovered by extraction of the lipids with chloroform-methanol (Kanfer & Kennedy, 1963) or with butan-1-ol (Wright, Dankert & Robbins, 1965). An advantage of [$2\text{-}^3\text{H}$]glycerol as a lipid precursor is that if it is metabolized after phosphorylation rather than incorporated into lipids it is oxidized to dihydroxyacetone phosphate and the ^3H atoms are lost. In some experiments [$1\text{-}^{14}\text{C}$]glycerol was used as a precursor. Lipids were then extracted from the bacteria by the method of Kanfer & Kennedy (1963). The extract was dried on to planchets for counting in an IDL Lobetamat instrument.

Fractionation of cultures according to cell size. After a period of pulse-labelling, exponentially growing cultures of *E. coli* (20 ml. at a density of about 0.1 mg./ml.) were poured

Fig. 1. Synchronous growth of *B. megaterium* and *E. coli*. The cultures were starved of threonine and methionine respectively and the amino acids were restored at zero time. The hatched areas indicate periods of cell division. *B. megaterium*: Δ , dry weight; $\circ$, cell count. *E. coli*: $\blacktriangle$, dry weight; $\bullet$, cell count.

into an equal volume of crushed frozen medium containing unlabelled glycerol (5g./l.) and the cells were rapidly harvested by centrifugation in the cold. The pellets were gently resuspended in 0.5ml. of ice-cold 0.1M-tris-HCl buffer, pH 7.4, at 20°, and carefully layered on to 20ml. linear sucrose gradients [5–20% (w/v) sucrose in 0.01M-tris-HCl buffer, pH 7.4] in cellulose nitrate tubes. After centrifugation for 15 min. at 1000g in an International refrigerated centrifuge, the tubes were punctured at the bottom and fractions (1ml.) were collected in test tubes containing 0.02ml. of 40% (w/v) formaldehyde solution. For each fraction measurements were made of extinction at 600nm., refractive index (n_D^{20}) by using a refractometer (Bellingham and Stanley Ltd., London, N.15) and a sodium-vapour lamp, and total cell count. A portion was then taken for precipitation with trichloroacetic acid (5%, w/v) and determination of incorporated radioactivity as described above. The measurement of n_D^{20} permitted the extinction to be corrected for the refractive index of the sucrose and hence converted into bacterial dry wt./ml. The correction was made by using curves obtained by suspending known concentrations of cells in sucrose solutions.

For sucrose concentrations up to 20% (w/v) it was observed that a graph of extinction against $(n_D^{20})^2$ gave a straight line of negative slope, in agreement with theoretical expectations (Koch, 1961).

RESULTS

Synchronous growth. Fig. 1 illustrates the patterns of synchronous growth obtained with the two bacterial strains. After restoration of the required amino acid, dry weight increased exponentially, sometimes after a short lag with *B. megaterium*. The doubling time was invariably the same as that recorded before starvation. The cell numbers increased discontinuously, doubling at intervals roughly equal to the mass-doubling time. After the second division synchrony decayed rapidly, and measurements were not made beyond this point. Periods of cell division are marked with hatched areas in this and subsequent figures.

Incorporation of glycerol. When [2-³H]glycerol was added to unsynchronized cultures of *B. megaterium* and *E. coli* growing exponentially, differential plots of incorporation/ml. of culture against bacterial dry wt./ml. yielded straight lines (Fig. 2). Further, the curves could be extrapolated back to the point at which the glycerol was added.

Fig. 3 shows the results of an experiment in which [2-³H]glycerol was added to a synchronously growing culture of *B. megaterium*. Growth is indicated in the lower part of the figure by the exponential increase of dry wt./ml. Cell counts have been omitted for clarity, but periods of cell division are indicated by hatched areas (cf. Fig. 1). The lower curve (●) represents the radioactivity incorporated/ml., the label having been added at the point shown by the arrow. To present the results more clearly the slope of this curve was

Fig. 2. Incorporation of [2-³H]glycerol by unsynchronized cultures of *B. megaterium* and *E. coli* growing exponentially. [2-³H]Glycerol (0.1 μ C/ml., 500 C/mole) and unlabelled glycerol (0.5 μ g./ml.) were added to cultures growing with aeration at 37° when the cell concentrations were equivalent to 0.11mg. dry wt./ml. (*B. megaterium*) and 0.05mg./ml. (*E. coli*). Samples (1ml.) were taken at intervals into trichloroacetic acid (5%, w/v), and the radioactivity was determined. ○, *B. megaterium*; ●, *E. coli*.

calculated over small intervals of time (5 min.) and divided by the average value of the dry wt./ml. over each interval to yield a number that is plotted in the upper half of the figure and represents the rate of incorporation/unit wt. of cells. The value was approximately constant between divisions but showed a large transient increase at the time of the first cell division. The pattern was repeated in the second cycle but was less marked because of the decay of synchrony. The decrease observed thereafter may have been caused by the culture reaching a rather high density or by the glycerol approaching exhaustion.

The results of a typical experiment with *E. coli* are shown in Fig. 4. [1-¹⁴C]Glycerol was added at zero time to the synchronous culture, the growth of which is illustrated in Fig. 1. The lower curve in Fig. 4 shows the incorporation/ml. and the upper curve the rate of incorporation/mg. of cells, calculated as described above. Although the additional manipulations involved in lipid extraction resulted in greater scatter of the points, the overall pattern was clearly similar to that observed with *B. megaterium*. In addition, the decay of synchrony towards the second division was more marked with *E. coli*.

A few experiments were also carried out with

Fig. 3. Incorporation of $[2\text{-}^3\text{H}]\text{glycerol}$ by a synchronized culture of *B. megaterium*. Synchronous growth was initiated by adding threonine at zero time. Δ , Growth curve; cell division took place in the hatched areas, as judged by cell counts. $[2\text{-}^3\text{H}]\text{Glycerol}$ ($0.1\text{ }\mu\text{C/ml.}$) and unlabelled glycerol ($0.5\text{ }\mu\text{g./ml.}$) were added at the point shown by the arrow. The lower curve ($\bullet$) shows the incorporation/ml. of culture. The slope of this curve was determined over 5 min. intervals and divided by the mean value of the dry wt./ml. for each interval to give the rate of incorporation/unit weight of cells ($\circ$).

Fig. 4. Incorporation of $[1\text{-}^{14}\text{C}]\text{glycerol}$ into the lipid fraction by a synchronized culture of *E. coli*. Synchronous growth was initiated by adding methionine at zero time. Growth of the culture is illustrated in Fig. 1. The hatched areas indicate periods of cell division. $[1\text{-}^{14}\text{C}]\text{Glycerol}$ ($0.075\text{ }\mu\text{C/ml.}$, 23.6 c/mole , no added carrier) was added at zero time, and 1 ml. samples were taken for measurement of incorporation into the lipid fraction ($\bullet$). The upper curve ($\circ$) shows the rate of incorporation/unit weight of cells, calculated as described in Fig. 3.

both *B. megaterium* and *E. coli* with $[^{32}\text{P}]\text{orthophosphate}$ as a lipid precursor. The kinetics of incorporation into the lipid fraction were essentially identical with those recorded with glycerol.

The patterns of glycerol incorporation during synchronous growth should be compared with the constant rates of incorporation/mg. of cells obtained with unsynchronized control cultures (Fig. 2).

Fractionation of cells according to size. An unsynchronized culture of *E. coli* growing exponentially was pulse-labelled for 3 min. (0.06 generation) with $[2\text{-}^3\text{H}]\text{glycerol}$, and harvested and fractionated by sucrose-gradient centrifugation as described above. The results of the experiment are presented in Fig. 5. The corrected extinction values permit the distribution of bacterial mass in the fractions to be estimated, and from cell counts (which are omitted for clarity) it is possible to calculate the average mass/cell for each fraction. This quantity increased twofold from the top to the bottom of the gradient. The high value in the first fraction was probably caused by the small number of cells that failed to separate after cell division. The radio-

activity/ml. divided by dry wt./ml. is plotted as the top curve ($\bullet$). Since the culture was pulse-labelled this number is equivalent to the rate of incorporation/mg. of cells and is highest in the largest and the smallest cells, i.e. in those undergoing division at the time of labelling. This experiment therefore leads to the same conclusion as was drawn from the experiments with synchronous cultures, namely that the rate of glycerol incorporation (and therefore probably of lipid synthesis)/unit mass of cells shows a sharp increase at the time of cell division.

It was not possible to use the gradient technique to investigate the cell cycle in *B. megaterium* because the cells grow in chains of variable length and the centrifugation does not give a separation according to cell age.

Fig. 5. Fractionation of pulse-labelled *E. coli* cells according to size. An unsynchronized culture of *E. coli* growing exponentially (20 ml. at a density of about 0.1 mg./ml.) was labelled for 3 min. with $[2\text{-}^3\text{H}]\text{glycerol}$ (1.25 $\mu\text{C}/\text{ml.}$; no added carrier). The bacteria were harvested and centrifuged through a sucrose gradient, and fractions were collected as described in the text. For each fraction measurements were made of E_{800} , n_D^{20} , cell count and incorporation of radioactivity into a 0.5 ml. portion (○). The extinction was corrected for the refractive index of the sucrose, and hence dry wt./ml. was determined (△). From the cell counts (not shown) the mean dry wt./cell was calculated (▲). The incorporation/unit weight of cells is also shown (●).

DISCUSSION

The results presented in this paper indicate that the rate of incorporation of glycerol into the lipid fraction of *B. megaterium* and *E. coli* varies considerably throughout the cell duplication cycle, and in particular a large transient increase manifests itself at the time of cell division. Since this phenomenon does not appear to be an artifact of synchronization it seems reasonable to suppose that it is related to the mechanism of cell division. Lubochinsky & Burger (1969) described similar cyclic variations of $[^{32}\text{P}]\text{orthophosphate}$ incorporation into phospholipids in synchronized cultures of *Bacillus licheniformis*.

Since unsynchronized cultures incorporate glycerol into the lipid fraction at a constant differential

rate without any appreciable lag, the rate of incorporation is proportional to the rate of lipid synthesis. It is not possible to estimate how closely lipid synthesis can be identified with membrane growth, since lipids account for only about 25% of the weight of the membrane (Salton, 1967).

The lower, approximately constant, rate of lipid synthesis observed during the greater part of the cycle may represent membrane growth during cell elongation. Since to a first approximation rod-shaped bacteria retain a constant diameter and grow exponentially in length (Marr, Harvey & Trentini, 1966), the surface area of the cells also increases exponentially. Hence the rate of increase of surface area/unit cell mass might be expected to be approximately constant during interdivision periods.

The increased rate of lipid synthesis at the time of cell division may similarly be an indication of septum formation. Jacob, Ryter & Cuzin (1966) presented some evidence that membrane synthesis in *Bacillus subtilis* is restricted to an equatorial band around the cell. It is not yet possible to say whether the early events of septum formation involve simply an increased rate of membrane synthesis in this region, causing the membrane to pucker away from the rigid cell wall, or whether a different type of membrane is laid down and grows across the cell. In the latter case the present results would suggest that this membrane has a higher lipid content and therefore that the polar membrane differs chemically from that covering the major part of the cell.

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