DNA replication: the 30th anniversary of the bacterial model and the 'baby machine'

I first met Charles E. Helmstetter ('Chick' to all who knew him back then) in July 1963, when we both arrived in Copenhagen for postdoctoral studies in Ole Maaløe's laboratory. He had come from the NIH where he had been a postdoc in Don Cummings' laboratory. There he developed a new method to synchronize bacteria – the membrane-elution method, or as it is now called, affectionately, the 'baby machine'[1]. Helmstetter was in Copenhagen to introduce this method into Ole's lab and to study biosynthetic patterns during the division cycle. Ole's interest in synchronization was stimulated by his association with Max Delbruck and the Phage School[2].

I had just graduated from the Rockefeller Institute after four years with Norton Zinder studying the growth and biochemistry of f2, the male-specific RNA phage. I had decided to go to Copenhagen after reading the Schaechter-Maaløe-Kjeldgaard papers, about which I have written elsewhere[2]. I did not really grasp the elegance of those papers in 1963 – it took me nearly 30 years to finally understand them[3].

Helmstetter's earlier PhD work at the University of Chicago was on the effect of UV light on bacteria during the division cycle. At Chicago, the filter paper method was used to synchronize bacteria[4]. A pile of filter papers were placed in a holder, a bacterial culture was filtered through the papers and the initial filtrate was collected. The smaller newborn cells were assumed to trickle preferentially through the papers and enrich the initial drops. Cells in these drops were then resuspended in fresh medium with a synchronized culture as the assumed and expected result.

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Chick continued his bacterial synchronization studies at the NIH where a new technical development – the Coulter Counter – propelled his work forward. This electronic instrument counted and sized bacteria quickly and accurately, without requiring colony formation. As it turned out, the filter paper method did not really synchronize bacteria at all. Incorporation studies with the filtration method gave an exponential pattern for thymidine synthesis during the division cycle[4], an experimental indication that no selection or synchronization was being performed by the filter papers. As we were to eventually learn, the true pattern of synthesis during the division cycle is far from exponential.

Surprisingly, the eventual development of the baby machine method for cell synchronization was not based on the principle of small cells filtering through filter papers, but on an entirely different principle, the growth of cells bound to the filter. The idea that the cells were growing in the filter paper stack came about during a chat with a few people at a Biophysical Society meeting (including Dave Freifelder and Phil Hanawalt). Someone asked Chick how long the cells were filtered. He answered, 'a few minutes'. Someone replied, 'then they must be growing in the filter'. The conversation did not go much further that day, but that night an event took place that is reminiscent of Kekule's vision of snakes rolling about with their tails in their mouths (which was the famous inspiration for the structure of benzene). Chick lay in bed in the dark hotel room and stared at the ceiling. He began thinking about things being attached to the ceiling and growing. For unknown reasons he thought of chickens; if a hen were attached to the ceiling, the eggs would fall down. He realized this would be the case with the cells, went back to the NIH and did the experiment – long-term elution from a small stack of paper filters – and it worked.

When the cells that were attached to the filter papers divided, only newborn cells were released by division. One daughter cell remained attached to the filter and the sister cell was released. The final step in the development of the baby machine was the change to a single cellulose nitrate membrane filter to which the bacteria adhered after filtration. This gave the best synchrony, and is still the method used today[1]. Because only newborn 'baby' cells are released from the membrane, the method is called 'the baby machine'.

One of Ole Maaløe's main interests was macromolecular synthesis during the division cycle. Ole had studied DNA synthesis ten years before, but this work was marred by a major artifact. Using temperature-shock synchronization, Ole and Karl Lark found a DNA synthesis pattern in prokaryotes similar to that found in eukaryotic cells (a gap, then DNA synthesis and then another gap)[5]. Control experiments[6] (recounted in an earlier history[3]) showed that this result was not natural, but was caused by a synchronization artifact.

We North Americans (there were five of us) had a good year in Copenhagen. Every afternoon tea was set out in the library, whether or not you showed up, so we all planned our experiments around tea time. And this is where most discussions took place. On Thursdays, the lab attendant would approach a postdoc and say 'Har du penge?' – Do you have the money? Someone was obliged to pay for the Wienerbrød (Danish pastry) for that day. Other discussions took place in the little cafeteria over frikadiller smørrebrød (Danish hamburger on buttered bread) and chocolate with butter sandwiches.

But all good things soon end. After a year, Chick accepted a position at Roswell Park Memorial Institute in Buffalo. I moved to Hammersmith Hospital in London, England, to William Hayes' Genetics Department. (In Copenhagen, I became interested in the genetics and biochemistry of D-methionine use[7], and the Hayes' group was a superb bacterial genetics lab.) Chick continued to work on the membrane-elution method while at Roswell. I spent the year 1964–65 in London, then moved to Tufts Medical School in Boston, USA, to work on protein synthesis with Kivie Moldave whom I had met in Copenhagen.

Chick was not satisfied with his synchronization procedure. At Roswell he worked on every detail of the method. He obtained special cabinets with heat curtains that allowed experiments to be performed at a constant temperature without a warm room. He looked at uridine, uracil and thymidine incorporation. After two years, he came to the realization that his beloved baby machine had problems. The baby machine still perturbed cells. Even a slight change in temperature led to measurable perturbations in incorporation, and such changes were certainly occurring on the membrane. He put special thermocouples on the membrane surface to measure the surface temperature. He used dyes to analyse fluid flow across the membrane surface. But in the end, the perturbations could not be eliminated.

In frustration, and unable to sleep, Chick spent another night thinking and worrying about the method. He decided that the method had failed. As with other synchronization methods, his method did perturb cells. What could he salvage from the method he had worked on for over five years? What could he change, or modify? He gazed out the window to the snowy landscape. He went over, in his mind, all possible permutations of the experiment. The usual experiment was to put cells on the membrane, collect the cells and then label cells at different ages. Hmmm! What if the cells were labeled first, then put on the membrane? He realized he had solved the problem. The newborn cells that were eluted from the bound cells would be eluted in reverse age order at division. The newborns first off the membrane after binding would be from the *oldest* cells in the culture (those just about to divide). With time, the newborns would come from *younger and younger* cells. By measuring the radioactivity per cell in the eluate during elution, the radioactivity incorporated into the original, unperturbed cells could be determined as a function of cell age.

There was a standard 'post-elution labeling' experiment planned the next morning. Though tired from a sleepless night, Chick went to the lab and told his technician to change the protocol. Label the cells first, bind the labeled cells to the membrane, and then analyse the radioactivity per cell in the eluate. Although the movie version would be better if the experiment worked that morning, the first experiment was a failure because too much radioactive thymidine was added.

The next day the experiment was repeated with less thymidine, and it worked. It worked beyond all expectations. The plateaus and dips were clear. The DNA replication pattern during the division cycle was obvious. And he had a method; the backwards method was born[8].

Chick wrote to me at Tufts shortly after performing this experiment. He explained the results obtained with moderate- and slow-growing cells. The graphs were extremely clear. At the beginning of elution there was a clear 'dip' in the radioactivity per cell. These were slow-growing cells, and the results indicated a synthetic gap in the older cells of the division cycle, a 'G2-phase' in eukaryotic terminology.

When I received the letter I was filled with excitement. I sat down and wrote a

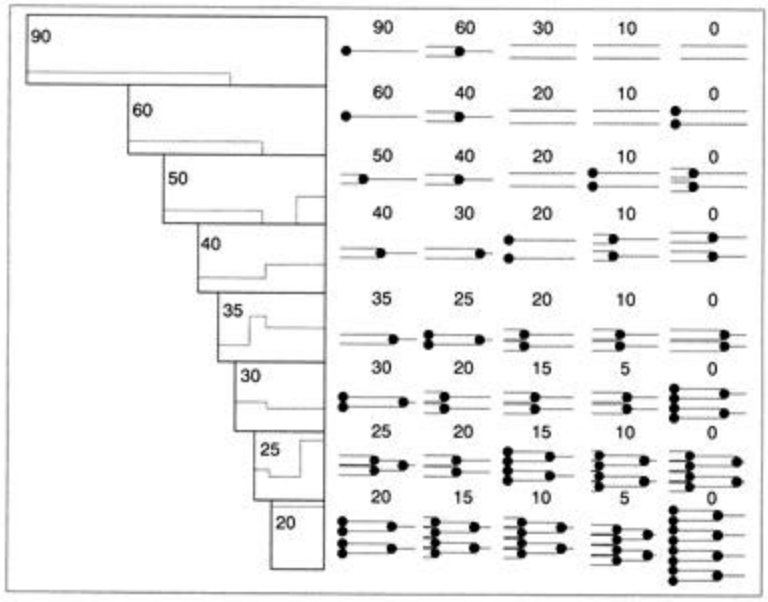

Figure 1

DNA replication patterns during the bacterial division cycle as originally understood in 1968. At the left are the proposed rates of thymidine incorporation for the chromosome configurations illustrated at the right. The numbers above the chromosomes indicate the number of minutes before a division. Between 20- and 60-minute interdivision times (numbers in the rate diagrams), a round of replication takes 40 min, and the time between termination and cell division is 20 min. A complete description of the derivation of these figures is given in Ref. 3.

43-page, handwritten letter (dated 14 January 1965, but obviously 1966) discussing the experiment (this was before computers!). My first sentence read: 'Your last letter was belated, long, exciting, and if I may be allowed – brilliant'. I continued, 'I think you have hit on something that may make the "selectostat" famous and also answer some very interesting questions' (the 'baby machine' name had not yet been invented and so I did not know what to call it).

Most of the letter related to the segregation problem, which at that time was one of the major problems in bacterial growth. I included drawings of different segregation possibilities, wrote about different models, and analysed various experiments with the pre-labeling method. Besides the segregation problem, I wrote about enzyme synthesis during the division cycle[9]. I also speculated about cell wall growth and drew a hypothetical picture of diaminopimelic acid (a wall-specific label) incorporation during the division cycle[10].

The amazing part of the story comes with Robert Guthrie's entry into the picture. Robert Guthrie was the developer of the 'Guthrie Test', a test for phenylketonuria (PKU) in newborn infants. Guthrie, at the University of Buffalo, had developed a simple test whereby a drop of blood from a baby is placed on an absorbent card, the card is mailed to a central laboratory, and a bacterial bioassay measures the presence of phenylketones. If a child were found to have PKU, the child was given a special diet, low in phenylalanine, and mental retardation was prevented.

In 1966, Kivie Moldave was offered a position as Chair of Biochemistry at the University of Pittsburgh. I decided not to go with him but to look for another position. I answered an advertisement in *Science*, and arranged to meet Guthrie at the annual Biochemical Society meeting. Because of my work on D-methionine metabolism (begun in Copenhagen, continued in London and finished at Tufts), Guthrie felt that I could extend the PKU test to other blood-testable inherited diseases. And so I moved to Buffalo in the summer of 1966. My office was temporarily occupied, and I had a month to kill before I could be fully at home at Children's Hospital where the PKU laboratories were located. What else was there to do but to go over to Roswell and work on DNA replication?

Chick had just finished analysing slow-growing cells. He had shown that the gap became more distinct at slower and slower growth rates. In the fastest cells he studied, glucose-minimal grown cells, there was no gap. A round of replication took 40 min. Serendipitously, this explained Ole's control experiments showing no gap in DNA synthesis[6].

When I arrived, it was logical and obvious that we ought to look at faster growth rates. We did the thymidine labeling on glucose–casamino-acids cells and the labeling pattern was the same as in 40-minute cells. Aha! We had it. Cells growing slower than 40-minute doubling times would have a gap, because the doubling time was greater than the time for a round of replication. With faster growth rates, the DNA replication rate sped up so a round of replication was equal to the doubling time. This model proposed that in 20-minute cells the pattern of replication would look exactly like that of the 40-minute cells; the time for a round of replication would be 20 min rather than 40 min.

That night I went home and wrote a full paper describing the model. The wonderful thing was that essentially all the available data fitted the model. I was able to fit the famous Cairns picture (an autoradiograph of a single *Escherichia coli* chromosome caught in the act of replication), run-out experiments (determining the amount of residual DNA replication following inhibition of protein synthesis), and all sorts of little pieces of published data into the general model. As it turned out this model was not correct. All of this external data had no ability to discriminate between the model I wrote up that night and what was eventually the correct model.

Of course the only experimental result that did not fit was the most important one. In what I have called 'The Fundamental Experiment of Bacterial Physiology'[2,3], Schaechter, Maaløe and Kjeldgaard found a continuous variation in DNA content as growth rate was varied in different media. (I was familiar with this experiment, because it was the one that persuaded me to go to Copenhagen.)

The DNA content was highest in fast-growing cells and lowest in slow-growing cells, and each growth rate had a different DNA content. The model we first considered had the same DNA content for all cells growing faster than a 40-minute doubling time.

Karl and Cynthia Lark had proposed a model to explain the different DNA contents in cells growing at different rates. They proposed that glucose-grown cells had two chromosomes replicating simultaneously, and that slower succinate-grown cells had two chromosomes replicating sequentially[11]. The Larks proposed that the glucose-grown cells were expected to have more DNA than the succinate-grown cells, thus solving the DNA content problem. As we wrestled with this problem, it became apparent that the Larks' model had identical DNA contents in both glucose- and succinate-grown cells. Two chromosomes replicating simultaneously throughout the division cycle had the same cellular DNA content as cells with two chromosomes replicating sequentially.

Over the next few days, Chick and I continued doing experiments at other growth rates. As the method was improved, and as results became sharper, we noticed a 'peak' near the step in incorporation. As we now understand it, this peak was a result of multiple-fork replication. Careful analysis of the results indicated that there was a constancy in time between the termination step and division and a constancy between initiation and termination. I rewrote the paper, using the same external data, to propose a constant 40 min for a round of replication (C period) and a constant 20 min between termination and division (D period). Cells growing faster than 40-minute doubling times had periods with multiple forks for DNA replication. The patterns of DNA replication during the division cycle are illustrated in Fig. 1. Three years earlier, at a cafeteria lunch in Copenhagen, I heard Ole talk about the rumored results of Yoshikawa and Sueoka on multi-fork replication in *Bacillus subtilis*[12]; this result certainly aided in understanding the *E. coli* results.

Now the fun began. I estimate that we went through approximately 33 drafts of the two papers that were eventually published in the *Journal of Molecular Biology*[13,14]. Drafts went back and forth each day – without the benefit of Email or fax machines. Chick's attention to detail matched mine, and together we worked on every word. I redid DNA measurements to determine the molecular weight of the *E. coli* genome. As it turned out, the results were remarkably close to the current value based on the DNA sequence. This indicated that the constant C and D model was correct, that the chromosome configurations we drew (Fig. 1) were correct, and furthermore, that the membrane-elution method could be used to determine synthetic patterns during the division cycle.

Photograph from the 1968 Cold Spring Harbor Symposium on Replication of DNA in Microorganisms. Front row: K. G. Lark, M. Schaechter, S. Cooper, O. Maaløe; Back row: P. Hanawalt, J. Clark, C. Levinthal, J. Watson, P. Kuempel, C. Helmstetter, D. Glaser.

I began experiments on the shift-up (the other part of the 'Fundamental Experiment'), looking at DNA replication patterns as cells were shifted from a slow-growth medium to a fast-growth medium. Because the Coulter Counter allowed many more experiments to be performed, the rate-maintenance phenomenon observed by Schaechter, Maaløe and Kjeldgaard (continuing the pre-shift rate of cell division for 60 min after shift-up) was confirmed for many different shift-up combinations. The complexity of the thymidine incorporation during a shift-up was difficult to fathom until I began a computer analysis with Steve Margolis, in which we simulated the shift-up conditions using the constant C and D periods and the constant initiation mass model[15]. Plotting the results revealed an amazing result. At 60 min of elution (a C + D period), there was always a drop in radioactivity per cell from the cells eluted from the baby machine. The explanation became clear. We did not need the computer any more. Rate maintenance was explained by the invariant Cs and Ds even during a shift-up. Newly inserted replication points could not determine a new cell division until at least a C + D period (60 min) had passed[16].

The papers we published broke naturally into two parts. The first paper dealt only with the observed pattern of DNA incorporation during the division cycle; there was a minimum of interpretation. The second paper interpreted the incorporation data with chromosomal replication patterns. In this second paper, the DNA contents I had measured were used to calculate the molecular weight of the *E. coli* genome. This molecular weight calculation was done in the simplest and most straightforward way possible: the amount of DNA was determined for a known number of bacterial genomes, giving the molecular weight directly.

Our papers were published in 1968 (Refs 13, 14) and later that year we presented a paper at the Cold Spring Harbor Symposium[17]. In that Symposium paper, a major generalization was unveiled, as we reported the remarkable relationship of our C and D periods to the eukaryotic S and G2 periods. As growth rates varied, the C and D periods, and the S and G2 periods in eukaryotic cells, remained constant. This was the initial finding that led to the continuum model[3,17–19], about which more next year, its 30th anniversary.

Although I left Buffalo in 1970 to move to the University of Michigan, the collaboration begun in 1966 or even in 1963 still continues. This year is the 30th anniversary of the initial description of the baby machine and the pattern of

DNA replication in slow-growing cells[8]. That result, and in particular the backwards membrane-elution method, generated many other results. Some are well known and appreciated; others are not well known. The segregation problem was illuminated by the baby machine as we now know that individual DNA strands segregate non-randomly at division[20]. In addition, the pattern of cell wall synthesis during the division cycle (also a topic of that first letter over 30 years ago) was worked out and understood using the membrane-elution method[10]. The same method was used to solve the controversy over the pattern of cell growth (linear or exponential)[21,22], and to clarify the pattern of segregation of the major cellular components[20]. Most interesting, the problem that started the entire collaboration, the segregation problem, was advanced by two separate membrane-elution approaches[23–26].

The most lasting effect of that time in Buffalo was having the feeling for a while that we were the only people in the world who knew something that no one else knew. I think it was best described by Albert Einstein who, upon gaining the insight of his General Theory of Relativity in 1915, said that 'something snapped inside me'. I too can say that I have felt that 'snap'. Even after 30 years I remember that feeling with joy and excitement. Yet best of all, I remember the wonderful collaboration that I had with my long-time friend and associate, Chick Helmstetter.

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