STUDIES ON DNA REPLICATION AND CELL DIVISION IN BACTERIA

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by

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INTRODUCTION

(I) Structure and Mode of Replication of the Bacterial Chromosome

The development of our knowledge about the mechanism of the replication of the genetic material in microorganisms started after Watson and Crick (1953) first proposed their model for the structure and possible mode of replication of DNA. The semiconservative pattern of DNA replication which they proposed was confirmed by Meselson and Stahl (1958). The bacterial chromosome was later shown to be a single circular molecule of double-stranded DNA genetically (Jacob and Wollman, 1961; Taylor and Adelberg, 1961) as well as physically (Cairns, 1963a,b; Bonhoeffer and Gierer, 1963).

Its sequential and oriented replication, suggested by Maaløe (1961) has been confirmed since then in many systems. Thus Lark *et al* (1963) were able to show, with the aid of density and radioactive isotopes of thymine, that a fragment of DNA in *Escherichia coli* 15T^{-} having once replicated is replicated again only after a large proportion of the chromosome has doubled. Autoradiography and electron-microscopy enabled Cairns (1963a,b) to reveal only one replication point on a chromosome of glucose grown *E.coli* B/r travelling sequentially along the chromosome with an apparently constant velocity. Yoshikawa and Sueoka (1963a,b) established that chromosome replication in *Bacillus subtilis* starts from a unique point on the chromosome (the origin) and proceeds in one direction. This was shown by measuring the relative frequencies of ten different markers in a steady state exponentially growing culture of this

strain (for a description of the method used, see Sueoka and Yoshikawa, 1965; and see also section (3)).

The pattern of duplication of λ prophage in synchronized cultures of two Hfr strains of E.coli K12 (λ) was interpreted by Nagata (1963a, b) as evidence for a polarity of chromosome replication. Finding a different pattern in F^- strains he concluded that a different mode of replication occurred in these strains. This last suggestion was not accepted by Jacob, Brenner and Cuzin (1963). Berg and Caro (1967) used generalized P1 transduction to assay relative gene frequencies in exponentially growing cultures of E.coli in a way analogous to that used by Yoshikawa and his colleagues for B.subtilis. Their conclusion supported that of Jacob et al (1963). Contradicting Nagata's results, they claimed that the site of integration of the F factor had no effect on the origin or direction of chromosome replication.

Exploiting the preferential mutagenic action of N-methyl-N'-nitro-N-nitrosoguanidine (NG) on regions of the chromosome engaged in replication, Cerdá-Olmedo and Hanawalt (1968) developed a method for the study of various aspects of chromosome replication in synchronized populations of cells. Using this method they confirmed the above findings and concluded, that the origin of several unrelated strains of E.coli was situated at a map position of about 50 minutes and that the direction of replication was the same (clockwise by convention - see Taylor, 1970) in all of them. This was supported by Wolf, Newman and Glaser (1968) who used the P1 transduction technique to assay markers near the origin or near the terminus.

They selected these regions by density gradient ultracentrifugation of P1 prepared upon donor bacteria containing DNA prelabelled with BU (5-deoxybromouracil) immediately after, or during amino acid starvation, respectively. This treatment is believed to allow completion of replication cycles that are in progress at the time of the shift-down but to inhibit further initiations (Maaløe and Hanawalt, 1961; Lark _et al_, 1963; Hanawalt _et al_, 1961)

The sequential nature of observed changes of enzyme inducibility in synchronous cultures (Masters et al, 1964; Kuempel _et al_, 1965) has been suggested to be a consequence of sequential replication starting from a fixed point (Donachie and Masters, 1966), assuming a relationship between this parameter and the gene dosage of the corresponding structural gene in the culture (Jacob _et al_, 1960; Donachie, 1964). The results obtained by Pato and Glaser (1968) and by Helmstetter (1968) using this method, confirmed that in _E.coli_ B/r the replication of the chromosome is sequential and clockwise, starting from an origin which is located in the 6-9 o'clock quadrant of the genetic map (Taylor, 1970).

(II) Chromosome Replication Velocity

Soon after Abbo and Pardee (1960) argued for an accelerating velocity of chromosome replication throughout the cell cycle, Maaløe (1961) suggested that the rate of DNA synthesis in a bacterial culture is determined by (a) the velocity of the replication process itself and by (b) the frequency with which new rounds of replication are initiated, and that the rate of DNA synthesis in a culture after a shift-up to a richer medium might be increased by an increase in the frequency of initiations only, without any change in the replication velocity.

The importance of the phenomenon of "premature initiation" after a period of thymine starvation, which was revealed by Pritchard and Lark (1964), was its distinction between the two processes ((a) and (b)) suggested by Maaløe (1961). Together with "dichotomous replication" (or "multifork replication"), found by Yoshikawa et al (1964) in fast growing bacteria, these two observations proved the concept that an initiation event is independent of completion of the previous round. Multifork replication in fast growing cells was later confirmed by Ward and Glaser (1969). Oishi et al (1964) explained this phenomenon as the consequence of a fixed replication velocity at individual forks of the chromosomes in rapidly growing cultures.

A constant velocity of replication was first shown by Cairns (1963 a,b). In their monograph Maaløe and Kjeldgaard (1966) suggested again, that the replication velocity of DNA was constant throughout the replication cycle and independent of the growth rate of the culture. The development of an improved method for synchronizing cultures without affecting the cellular physiology by Helmstetter and Cummings (1963, 1964) enabled Clark and Maaløe (1967) to confirm this concept. The rate of DNA synthesis, measured by pulse labelling with radioactive thymidine of a synchronized culture of E.coli B/r growing at several rates, was constant throughout a large part of the cell cycle. They suggested that discontinuities in this rate, which occurred at specific times in the division cycle (specific to each growth rate) were due to the initiation or completion of cycles of replication.

These conclusions were most clearly demonstrated soon after by Helmstetter and Cooper (1968) and Helmstetter et al (1968) using the

same synchronization technique. They concluded that the replication velocity of the chromosome of E.coli B/r was constant over a wide range of doubling times ($20 \leq \tau \leq 65$ minutes) and constructed a model consistent with their data (Cooper and Helmstetter, 1968), that connects bacterial cell division to chromosome replication. Briefly, *their model can be stated in terms of two parameters: C, the time* required for a replication point to proceed from one end of the genome (origin) to the other (terminus), and D, the time between the end of a round of chromosome replication and subsequent cell separation. They determined the replication time (C) in E.coli B/r to be 46 minutes and D to be 24 minutes (after correcting for the differences in growth rate between batch cultures and cells adhering to a membrane filter) for the above range of generation times. Three main consequences emerge from this: (a) cells growing slower than C minutes per doubling ($C < \tau$) exhibit a gap between rounds of DNA synthesis; (b) cells growing faster than C minutes per doubling ($C > \tau$) contain chromosomes with multiple replication forks for at least part of their division cylce; (c) cells growing at a rate C minutes per doubling ($C = \tau$) have no gap and no multiple forks.

Prediction (b) is an equivalent statement to the so called "dichotomous replication" mentioned before as revealed by Yoshikawa et al (1964) when they demonstrated multiple replication points on chromosomes of rapidly growing cultures of B.subtilis. Multiple fork replication was probably the reason for the bigger fractional increase in DNA (ΔG) in amino acid starved cells of E.coli $15T^-$

pregrown on broth medium compared to the corresponding increase in such cells after growth in a glucose medium as recorded by Schaechter (1961). Prediction (a) was also confirmed by several authors (e.g. Lark, 1966; Helmstetter, 1967) who showed that at slow growth rates a significant fraction of cells in a population is not synthesizing DNA.

Although it is generally accepted now that the replication time of the bacterial chromosome is constant in wild type strains, there were some contradictory results recorded in the literature when this study was started. The common feature of most of them is that they were obtained in thymine-requiring strains. A detailed review will be presented in the Discussion, so that they can be compared to the results obtained in this study and analysed in their light.

Two main observations led R.H. Pritchard to introduce a new hypothesis, namely, that the replication time of the chromosome in *thy*$^-$ strains depends on the external concentration of thymine present in their growth medium. Firstly, it appeared that such mutants were able to incorporate thymine into DNA only by maintaining a lower internal dTTP (deoxythymidine triphosphate) concentration than in their *thy*$^+$ counterparts (Beacham *et al*, 1968) and although this concentration was raised as the amount of thymine in the medium was increased, it could not rise to the normal level present in *thy*$^+$ strains (Beacham *et al*, submitted for publication).

The second observation drawing the attention to such an hypothesis was made by Friesen and Maaløe (1965). They pulse labelled several *thy*$^-$ strains of *E.coli* with a set of concentrations of ^{14}C-thymine and found in all of them a higher rate of incorporation of

the label into the DNA the higher the thymine concentration used. Friesen and Maaløe showed that the strains they used were not leaky, but they did not give any explanation for their finding. The increased rate of incorporation observed must have reflected an increase in the rate of DNA synthesis.

(III) Estimation of the Chromosome Replication Time - Theoretical Considerations

There are at least eleven methods, more or less independent of each other, by which the replication velocity of the bacterial chromosome (i.e. the reciprocal of the replication time, C) can be estimated. Only eight of them give C in absolute terms (minutes), while the other two give relative velocities. These are summarized in Table 1. The set of assumptions underlying these methods is given below. Any given method may involve only some of them.

(a) Initiation of a cycle of replication occurs when the cell mass per chromosome origin reaches a fixed value (Pritchard, 1968; Donachie, 1968; Pritchard *et al*, 1969).

(b) Completion of a cycle of replication provides a signal for cell division which occurs (C + D) minutes after initiation (Cooper and Helmstetter, 1968; Helmstetter *et al*, 1968; Helmstetter and Cooper, 1968; Helmstetter and Pierucci, 1968), where D is the time between completion of chromosome replication and the subsequent cell division.

(c) Replication forks traverse the chromosome with uniform velocity at steady states of growth (Cairns, 1963a; Clark and Maaløe, 1967).

(d) Removal of a required amino acid from the growth medium permits completion of cycles of replication that were initiated before amino

acid deprivation but inhibits further initiation (Maaløe and Hanawalt, 1961; Hanawalt _et al_, 1961; Lark _et al_, 1963; Caro and Berg, 1968; Wolf _et al_, 1968).

(e) Powell's age distribution function for cells,

$$f(x) = (\ln 2)2^{1-x}, \text{ where } 0 \leqslant x \leqslant 1 \text{ is cell age in fractions of generation} \quad (1)$$

is valid and describes also the age distribution of replication forks in steady state exponentially growing (s.s.e.g.) cultures (Powell, 1956; Sueoka and Yoshikawa, 1965).

(f) Thymine deprivation of a _thy⁻_ culture in s.s.e.g. for a period equivalent to one mass doubling does not alter the replication velocity when thymine is restored at the same concentration as that present in the growth medium prior to its removal.

(g) The replication of the bacterial chromosome is sequential and starts at a specific and constant point (origin), which may vary from one strain to another (Meselson and Stahl, 1958; Nagata, 1963a,b; Yoshikawa and Sueoka, 1963a,b; Berg and Caro, 1967; Cerdá-Olmedo _et al_, 1968).

The eleven methods summarized in Table 1 will now be discussed in more detail (see also Discussion).

(1) This was the first method used to determine directly the replication velocity of the bacterial chromosome (Cairns, 1963a). With the aid of autoradiography and electron microscopy he estimated that the velocity with which the chromosome of _E.coli thy⁻_ is replicated when supplied with thymidine is 27 μ per minute and constant. This velocity corrresponds to a replication time of 41-52 minutes (considering 1100-1400 μ as the total length of the chromosome).

TABLE 1. Summary of methods available to quantitate C

No.	Method	Reference(s)	Assumptions	C-values
1.	Direct	Cairns, 1963a.	None	Absolute
2.	Rate of DNA synthesis	Friesen & Maaløe, 1965; Pritchard & Zaritsky, 1970.	(a)	Relative
3.	Step-time	Manor (personal communication)	None	Absolute
4.	Fraction of unlabelled cells	Lark & Lark, 1965; Dennis & Herman, 1970.	(b), (e)	Absolute
5.a	HOT	Yoshikawa & Sueoka, 1963; Berg & Caro, 1967; Wolf *et al*, 1968.	(e), (g)	Absolute
5.b	F	Manor (personal communication); Ward & Glaser, 1969.	(e), (g)	Absolute
6.	Rate of DNA synthesis versus cell age	Helmstetter & Cooper, 1968.	None	Absolute
7.	ΔG	Maaløe & Hanawalt, 1961; Pritchard & Zaritsky, 1970	(c), (d), (e)	Absolute

8.	$\bar{G}$	Cooper & Helmstetter, 1968.	(b), (c), (e)	Absolute
9.	$\bar{M}$	Donachie, 1968; Pritchard et al, 1969.	(a), (b), (e)	Relative
(8-9)a	$\bar{G}/\bar{M}$	Pritchard & Zaritsky, 1970.	(a), (c), (e)	Relative
(8-9)b	$(\bar{G}/\bar{M})_1$ ——— $(\bar{G}/\bar{M})_2$	Pritchard & Zaritsky, 1970.	(a), (c), (e)	Absolute
10.	RSF	Pritchard & Lark, 1964; Zaritsky & Pritchard, in prep.	(a), (c), (e), (f)	Absolute
11.	FPC	Chai & Lark, 1967; Chan & Lark, 1969; Vielmetter et al, 1968.	(b), (e), (g)	Absolute

Although this is a direct method which does not depend on any of the assumptions mentioned above, it has some technical limitations. One factor that should be taken into consideration is the time taken to exhaust the internal "cold" pools of the thymine derivatives that are DNA-precursors. This time is strain-dependent and varies widely in different thy$^-$ strains of E.coli (Caro and Berg, 1968; Leighton & Donachie, 1970).

(2) The observation of Friesen and Maaløe (1965) provides the basis for the second method (and see previous section). They showed that various thy$^-$ strains of E.coli incorporated progressively more ^{14}C-thymine into their DNA when pulsed with progressively higher concentrations of this isotope. A similar finding in E.coli B/r thy$^-$ was reported by Freifelder (1965) soon afterwards. Since the growth rate of such cultures was not apparently affected it seemed reasonable to us to suppose that what was being observed was a transient increase in the velocity of DNA replication as a result of the increase in the concentration of thymine in the growth medium. If this were so, it could form the basis of a technique for measuring relative velocities independent of any of the assumptions listed above.

Two factors must be checked when employing this method: (i) the time taken to exhaust the "cold" pools of dTTP; (ii) the length of time in which the incorporation of the radioactive thymine into DNA is linear, since it starts to vary in a complicated fashion due to the deviation of the chromosomes from s.s.e.g. conditions.

(3) The third method which is independent of any of the assumptions presented above is to measure biochemically the step-time for the addition of nucleotides to the growing DNA chain in vivo. This was

used only recently by H. Manor (personal communication) and it seems that in E.coli B thy$^-$ this step-time is three-fold longer, using 0.2 µg/ml thymine, than it is when thymidine is used (Cairns, 1963a).

(4) Lark and Lark (1965) tried to measure the replication time of the chromosome of E.coli 15T$^-$ (555-7) of cultures growing at various rates by pulse labelling with radioactive thymine and determining the fraction of unlabelled cells. This procedure provides another method by which one can calculate C.

If assumptions (b) and (e) are valid the following statement may be made:

$$\text{fraction of unlabelled cells} = \int_{(\tau - D + t)/\tau}^{2-(C+D)/\tau} f(x)dx \qquad (2)$$

where t is the pulse time (in minutes). Since in all cases where $\tau \leq C$ every chromosome in the culture contains at least one replication fork and even after an infinitesimally short pulse all cells will be labelled, this method is applicable only when $\tau > C + t$. Moreover, the actual value of C calculated from it is dependent (to a slight extent) on two other factors: (i) the value of D; (ii) the time taken for the internal pool of dTTP to equilibrate with the radioactive material added.

(5a) The fifth method which can serve as a means by which to determine C is the ratio between the frequency of the most proximal marker to the origin and the frequency of the marker nearest to the terminus (HOT) (Yoshikawa and Sueoka, 1963a,b; Berg and Caro, 1967; Wolf et al, 1968). This ratio bears a simple relationship to the value of the "replication position" (n) of an average chromosome in the culture (Sueoka and Yoshikawa, 1965) such that

$$HOT = 2^n \qquad (3)$$

and since

$$n = C/\tau \qquad (4)$$

(Sueoka and Yoshikawa, 1965; Caro and Berg, 1969; Donachie, 1969; Pritchard and Zaritsky, 1970) it is a simple matter to deduce a value of C for any experimental values of HOT and τ. This principle served S. Borenstein and E. Elizur (personal communication) to show, with the aid of the transformation technique in _B. subtilis_, that C varies with the thymine concentration supplied to a _thy_$^-$ strain of this bacterium, as I have found in this study. This principle underlies also P1 transduction mapping in _E. coli_ (Wolf _et al_, 1968; Masters, 1970).

Assumptions (e) and (g) underlie this method. Its limitations in the case of assay by P1 transduction are due to the lack of markers near enough to the origin which are not behaving in a "puzzling" manner (see e.g. Masters, 1970) and to uncertainty that P1 transducing particles carry different genes in proportion to their relative frequency.

(5b) The same assumptions ((e) and (g)) also underlie the next method, which is, therefore not independent. From measurements of the average number of forks (replication points) per chromosome (F) one can calculate C, since

$$F = 2^n - 1 \qquad (5)$$

(see Appendix 1)

One of the ways to measure this parameter is by NG treatment (Ward and Glaser, 1969). H. Manor (personal communication) was able to measure F by direct biochemical methods.

(6) A sixth independent way by which a direct estimate of C (in minutes) can be determined is that applied by Helmstetter and Cooper (1968) for E.coli B/r. With the aid of the membrane-selection technique to synchronize cultures of this strain (Helmstetter, 1967) they measured directly the time taken for one round of chromosome replication to terminate (C). They found that this time was constant (C = 46 minutes) over a wide range of generation times ($20' \leq \tau \leq 65'$). Unfortunately this technique can be used only in the studies of few bacterial strains. Most strains do not apparently release populations of constant age from the filter. However, by special modifications Cummings (1970) and Shehata and Marr (1970) were able to synchronize certain other strains, thus extending the possible uses of this technique.

(7) The increment of DNA (ΔG) after removal of a required amino acid is another parameter, which can serve as a method to estimate C. Accepting assumptions (c), (d) and (e), this increment (in terms of percent of the DNA present at the time of the shift down) is given by the function (Sueoka and Yoshikawa, 1965)

$$\Delta G = \left(\frac{2^n . n . \ln 2}{2^n - 1} - 1\right) . 100 \qquad (6)$$

Since $n = C/\tau$ (Sueoka and Yoshikawa, 1965) then at a given growth rate, C uniquely defines ΔG (Fig. 1).

The possible errors implicit in this method are mainly due to assumption (d) which is not fulfilled in some cases. These will be considered more fully in the Discussion.

(8) Cooper and Helmstetter (1968) suggested that the average DNA contents per cell ($\bar{G}$) in a s.s.e.g. culture could be predicted according to their model. By assuming (b), (c) and (e) they derived

the following equation:

$$\bar{G} = \frac{\tau}{C \ln 2} \left(2^{(C+D)/\tau} - 2^{D/\tau}\right) \qquad (7)$$

and discussing the data available in the literature (Schaechter *et al*, 1958) they showed that this relationship holds true in some thy^+ strains of bacteria, when C and D were taken as 41 and 22 minutes, respectively.

However, considering the problem of quantitating C, this is a function with four variables, only two of which are easily measurable (i.e. $\bar{G}$ and τ). Moreover, the meaning of D in molecular terms and its quantitation are still very obscure. Since there exists the possibility that D is not independent of C (section (11) in the Results), $\bar{G}$ cannot be regarded as a valid method of determining C as long as the quantitation of D has not been cleared up.

(9) The same arguments are also true for another independent method for determining C. Assuming (a), (b) and (e) Donachie (1968) and Pritchard *et al* (1969) established the following relationship:

$$\bar{M} = k.2^{(C+D)/\tau} \qquad (8)$$

where $\bar{M}$ is the mean cell mass in a s.s.e.g. culture whose dimension depends on that of the constant k.

(8-9a) These two methods can be reduced to one, which is independent of D. Dividing (7) by (8) gives:

$$\bar{G}/\bar{M} = \frac{\tau}{kC\ln 2} \left(1-2^{-C/\tau}\right) \qquad (9)$$

(Pritchard and Zaritsky, 1970). Hence changes in $\bar{G}/\bar{M}$ (the DNA/mass ratio in the culture) will be a function solely of C and τ and independent of possible changes in D (Fig. 1).

Since the dimension of $\bar{M}$, and therefore that of $\bar{G}/\bar{M}$, are dependent on a constant, k, which is not known (it is a function of the "mass at initiation", defined by Pritchard _et al_, 1969), changes of $\bar{G}/\bar{M}$ can give only relative values of C. To evaluate the absolute values of C in minutes it is necessary to define a reference point such that, for one set of conditions (i.e. growth rate and thymine concentration, in a study like that presented here) a value of C must be assumed (or known). Because the function relating $\bar{G}/\bar{M}$ to C is not linear the relative values of C obtained depend also on the validity of the absolute value assumed for one value of $\bar{G}/\bar{M}$. This means that the assumed value of C for the reference point defines also to some extent the relative C values.

(8-9b) However, a simple trick using this principle can help to estimate absolute values of C in minutes. Changing the concentration of thymine in the medium of a s.s.e.g. culture of a _thy_$^-$ strain causes an immediate change in the rate of DNA synthesis in the culture, without any detectable change in the mass doubling time due to the resulting change in the replication velocity of the chromosomes in the culture (Pritchard and Zaritsky, 1970). This change must be temporary and the rate of DNA synthesis will return to the previous rate when the distribution of replication forks equilibrates to the new value appropriate to the new replication velocity such that the chromosomes in the culture are again in s.s.e.g. conditions. This will happen when the last replication fork to start in the previous conditions completes its job. Per definition - the time taken is the new replication time, C.