Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

Mario Ageno, Anna Maria Salvatore

On the cell damage induced in Escherichia coli by a prolonged block of DNA synthesis

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **72** (1982), n.6, p. 394–403.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1982_8_72_6_394_0>

Biofisica. — *On the cell damage induced in* Escherichia coli *by a prolonged block of DNA synthesis* (*). Nota di MARIO AGENO e ANNA MARIA SALVATORE, presentata (**) dal Corrisp. M. AGENO.

RIASSUNTO. — Un attento esame delle curve di crescita di colture di *Escherichia coli* ottenute dopo trattamento con acido nalidixico per un tempo t_0 suggerisce un modello per spiegare la natura del danno irreversibile che si produce nelle cellule quando il blocco della sintesi del DNA viene protratto per un tempo anche di poco superiore ad un opportuno valore di t_0.

In particolare, si propone che il distacco (per tensione meccanica o azione enzimatica) del cromosoma in fase di replicazione dall'involucro cellulare sia responsabile delle divisioni « asimmetriche » osservate alla fine del periodo di blocco. In queste divisioni solo una delle due cellule figlie prodotte è ancora capace di riprodursi.

In a previous paper [1] we suggested a model to explain the shape of the growth curves of *E. coli* cultures in which DNA synthesis was blocked for a time interval t_0 by treatment with nalidixic acid (NAL).

These curves, though showing significant differences from strain to strain, always turn out to depend critically on the value of t_0.

Furthermore, for values of t_0 of the order of the interdivision time τ, when DNA synthesis starts again after the block, one observes one or two synchronous cell divisions following rounds of chromosome replication beginning immediately after the block is removed.

On the other hand, the old non-synchronized replicating forks seem to have disappeared, as the number of viable cells remains constant between the end of the block and the first synchronous division (Fig. 1).

We have interpreted this in the context of the Jacob, Brenner and Cuzin model (which we shall refer to, from now on, as the JBC model) for chromosome segregation in procaryotes [2], making in particular, the following assumption:

1) The origin of the chromosome is permanently attached to the cell envelope [3].

2) A duplication cycle of the chromosome starts with the doubling of the origin [4]. As the replicating forks advance along the chromosome the separation of the two origins increases, according to the JBC model, while they include the region of lengthening of the cell envelope.

3) A spacing of the origins of about two microns is sufficient to produce the complete segregation of the two new chromosomes. This requires that the duplicating chromosome always retain a compact structure which never unwinds if not locally [5]. Therefore, if the replicating forks are blocked while the origins

(*) Ricerca finanziata dal Consiglio Nazionale delle Ricerche.
(**) Nella seduta del 25 giugno 1982.

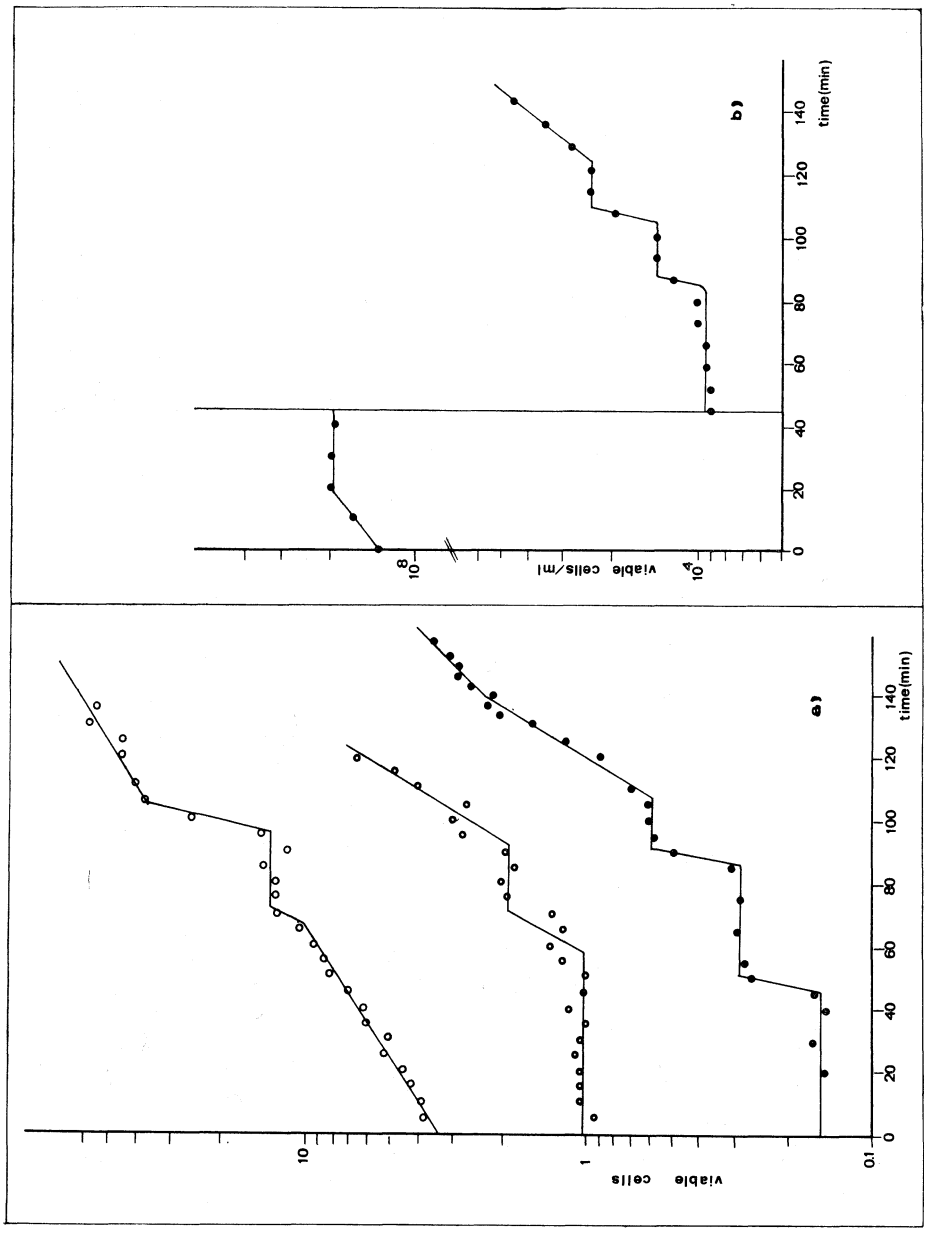

Fig. 1.

a) Growth curve of *E. coli* B after treatment with NAL for ○ 22,5 min; ● 27 min and ● 45 min.

b) Growth curve of *E. coli* K12 (W1485 F⁻) during and after treatment with NAL for 45 min.

keep separating from one another, the whole chromosome structure becomes tensioned and this may, at a certain point, conceivably produce irreparable cell damage.

In this paper we intend to re-examine our results carefully, in order to see to what extent the model is experimentally valid, and to find out, if possible, the nature of the cell damage produced by a prolonged block of DNA synthesis.

Let us start by considering the growth curves obtained for our strain of *E. coli* B, already reported in our previous paper and here summarized in Fig. 1. Let us recall that the culture medium was so selected as to make the following relationship approximately valid

$$\tau \simeq C \simeq 2\,D = 45 \text{ min} \tag{1}$$

where C is chromosome replication time and D time elapsing between the end of this process and the next cell division [6]. The validity of (1) means that, in a exponential growth regime, a new duplication cycle of the chromosome

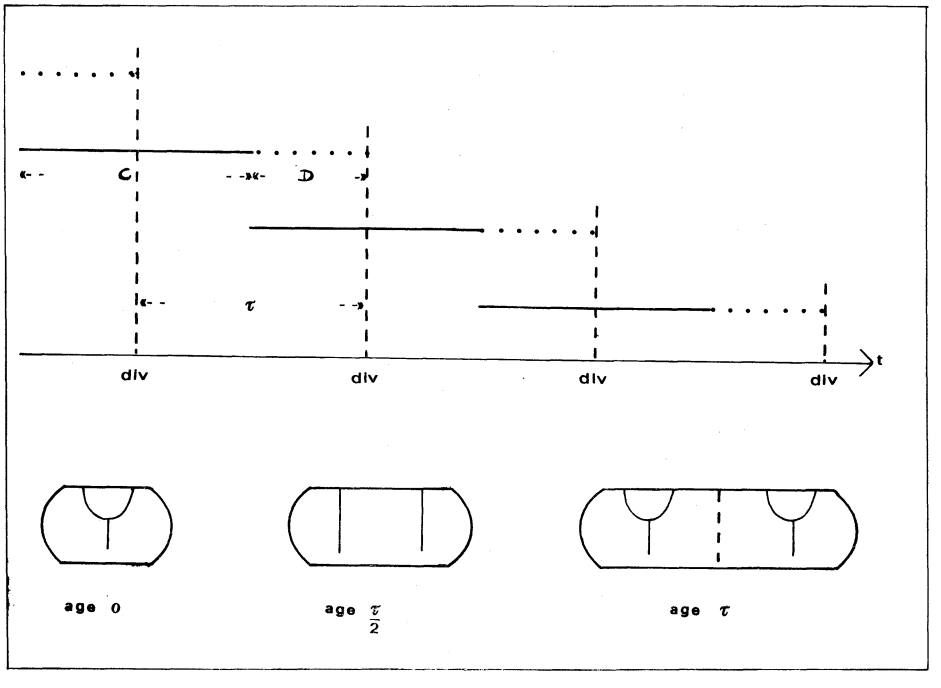

Fig 2. – Phase relations between chromosome duplication and cell division under the condition $\tau = C = 2\,D$.

starts as soon as the previous one ends and that the cell divisions take place when every chromosome has been half duplicated (Fig. 2). This makes the analysis of the growth curves after the end of the block easier.

The selected values of t_0 were: 22,5 min $= \tau/2$, 27 min and 45, min $= \tau$.

Irreversible cell damage appears clearly in the last two curves: they do not show cell division up to the first synchronous division, which evidently follows a round of DNA replication which started in all the culture cells immediately after the NAL was removed. The problem then arises of understanding what happened to the old non-synchronized replicating forks, blocked by the NAL for the whole period t_0 [7]. Should they start advancing again after the end of the block, they certainly would not be able to give rise to synchronous cell division.

It has been shown [8] that under particular conditions one effect of NAL is the destruction of DNA, starting from that more recently synthesized and always going on to that synthesized immediately before. One might then imagine that the old replicating forks have disappeared because of the destruction of the two strands undergoing synthesis. If so, only the parent strands would have remained in all the cells and, at the end of the block, DNA synthesis would start again from the origin, giving rise to a synchronous division. This assumption is to be excluded for two reasons.

a) If the duration of the block equals $\tau/2$, cell divisions go on for its complete duration (those cells which at the beginning of treatment had completed chromosome duplication divide under NAL). Then cell divisions start again immediately, after the elimination of NAL, with an interdivision time identical to that of the exponential phase preceding the block. This means that the old replicating forks advance again from exactly that point at which they had been blocked by the action of NAL. However, if the duration of the block is increased by only five minutes ($t_0 = 27$ min), the first cell divisions after the block are the synchronous ones. It seems very unlikely that sections of DNA strands, of all possible lengths between a half and a whole chromosome, remain unaffected by NAL for 22.5 minutes and are all equally destroyed in the following 5 minutes.

b) In the growth curves corresponding to higher values of t_0, after the more or less long initial phase of synchronous divisions exponential growth starts again. There are, therefore, in this phase, some non-synchronized replicating forks which advance regularly, starting from any intermediate point in the chromosome. It seems obvious that the starting points depend on the positions assumed by the old replicating forks at the moment when they were blocked by NAL. One is forced to conclude that the old forks are by no means lost.

But, if the old replicating forks are not lost, when the NAL is removed they must start to advance again along the chromosome, giving rise to non-synchronized cell divisions for which, however, there is no evidence in the curves of Fig. 1.

The only assumption capable of removing this contradiction is that the technique we used, namely colony counting, was not able to reveal non-synchronized cell divisions which presumably took place in the period preceding the synchronous ones.

In other words, after the elimination of NAL the old replicating forks should again start to advance regularly, also in the growth curves relative to the higher

values of t_0. Once chromosome replication is over, every cell should divide regularly: the irreversible damage produced by the prolonged action of NAL would reveal itself in the fact that one of the two daughter cells would be unable to form a colony on agar.

This argument prompted us to carry out for the same culture microscope countings of the total number of cells and colony countings of the viable cells. The results obtained for $t_0 = 45$ min, already reported briefly in our previous paper, are given here in Fig. 3. The two growth curves are far from being coin-

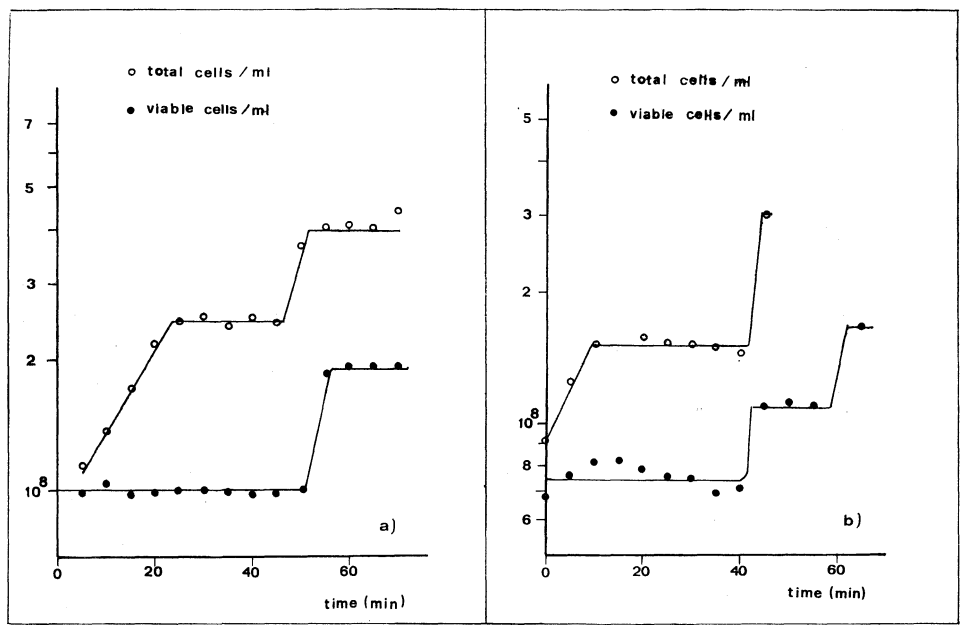

Fig. 3. – o Number of total and of • viable cells after treatment with NAL for 45 min in a) *E. coli* B and b) *E. coli* K12.

cident. Only the number of viable cells remains constant during the first 45 minutes, while one observes a steadily increasing fraction of cells incapable of reproducing.

After the first synchronous division the total number of cells is four times that of the cells present at the end of the block. However, only half of them are able to form colonies on agar. Every cell present in the culture at the end of the block has at this point undergone three divisions: two (asymmetric) divisions in which one of the two daughter cells is non viable, and one (normal) synchronous division.

We intend to reconsider elsewhere the process of asymmetric cell division as well as the detailed interpretation of the growth curves after the end of the block.

Here we wish to discuss in particular the nature of the cell damage produced by the prolonged action of NAL, as shown by these rather surprising

results. We wish to add that one must reject the supposition that the differences between microscopic counting and colony counting may be due to daughter cell association. Very accurate microscope observations showed that in our strains there is not an appreciable number of cells which remain associated after the division. On the other hand, it seems very hard to justify the exact quantitative relations in the growth curves of Fig. 1 and 3 with an assumption of this kind.

A simple inspection of the growth curves in Fig. 1 allows some important conclusions to be drawn:

1) The damage which gives rise to the non-viable cells does not seem to be due to a direct action of NAL. In fact it does not occur if RNA or protein synthesis is blocked at the same time as DNA synthesis; i.e. when the lengthening of the cell is hindered [9]. It then seems to be due to the phase-difference produced by NAL between the chromosome replication process and the other cellular processes which depend directly on protein synthesis, in particular the lengthening of the cell.

2) The damage we are interested in takes place in *no* cells, whatever the positions along the chromosome of the replicating forks may be, until the duration of the exposure to NAL exceeds a given minimum. In our strain of *E. coli* B this minimum is just greater than $\tau/2 = 22.5$ min. In fact, as has been already pointed out, in the growth curve relative to $t_0 = \tau/2$, the *normal* cell divisions start again immediately after the end of the block and there is no production of non-viable cells.

3) On the contrary, if exposure to NAL lasts a little longer than the minimum, this damage occurs immediately and at the same time in all cells in the culture. In our case, as already mentioned, an increase in t_0 of only 5 minutes is sufficient to completely change the shape of the growth curve after the end of the block: a long false plateau, along which the number of viable cells remains constant, while cells incapable of reproducing are generated by division, up to a first synchronous division in which every viable cell divides into two normal cells.

4) If exposure to NAL is prolonged, once damage has occurred in all the cells, no other cell damage is produced for a time which in our case corresponds to a value of t_0 even considerably greater than $\tau = 45$ minutes. After about one hour of exposure the number of viable cells, which was previously constant, starts decreasing rapidly. In particular, at least up to values of t_0 of the order of 60 minutes, after elimination of NAL, the cells are still all capable of dividing and (only) one of the two daughter cells behaves as a perfectly normal cell.

The cell damage produced by NAL seems then to be due to a *specific event of the "yes or no" kind*, which *always* takes place after an exposure even slightly longer than $\tau/2$ (in our strain).

On the one hand it has as the only consequence the incapacity of one of the two daughter cells to further divide and on the other hand it removes the state of uneasiness in which the cell finds itself owing to exposure to the drug.

It seems obvious to attribute this state of uneasiness to the mechanical tension induced in the chromosome structure by the phaseshift produced by NAL between the process of separation of the origins anchored to the cell envelope which keeps lengthening, and the advancing process of the replicating forks along the DNA, blocked by the NAL.

But, if this is really the cause of the uneasiness, its suppression through a single event of the "yes or no" kind may actually be obtained with the detachment from the cell envelope of (only) one of the two origins of the chromosome under segregation. An event of this kind removes the mechanical tension, decoupling the two processes of chromosome segregation and of DNA replication.

On the other hand, it evidently follows that when the NAL is removed the replicating forks can start advancing again and the subsequent cell division can take place normally, with the result, however, that (only) one of the two daughter cells has its chromosome detached from the cell envelope (or, possibly, has no chromosome if segregation did not occur regularly). It is incapable of starting DNA replication and then of further reproducing.

This assumption accounts for what we observed. However, one may wonder whether some alternative explanation exists. A possible explanation is that the removal of the mechanical tension is produced by means of two cuts effected in the already replicated DNA, in such a way that every replicating fork remains attached to only one of the two origins of the chromosome (Fig. 4).

Such an operation, however, can be performed in four different ways, two of which have as a consequence the survival of one of the daughter cells, whereas in the other two chromosome segregation turns out to be impossible, unless a further cut is effected in the DNA molecule. In this case both daughter cells would turn out to be incapable of reproducing. If these four modes were equally probable, the number of viable cells should reduce to half, instead of remaining constant, as is observed.

There seems to be no reason to leave out the two ways leading to the death of both daughter cells. On the contrary there might be some reasons to consider one of them more probable than the others. In fact, if the damage does not occur near the origin, it is conceivable that it should take place in the proximity of the replicating forks: in all the other regions the chromosome assumes a very compact structure if, as is supposed, the segregation takes place according to the JBC model. The two weaker DNA sections, which are the best candidates for a break, are those along which the synthesis proceeds by Okazaki fragments, which are, in fact, each connected to a different origin.

Now, it remains to be seen what *the mechanism* of detachment of the duplicating chromosome from the cell envelope may be. The simplest assumption is that this detachment is a direct effect of the mechanical tension generated by the block of the replicating forks, while the two origins of the replicating chromosome keep moving away from one another. A second assumption is that it occurs through the action of an enzyme which recognizes and cuts (or simply detaches from the wall) the DNA under tension.

The idea of purely mechanical damage is certainly to be considered, since it can very simply account for all the aspects of the phenomenon. Furthermore, there is evidence that the point of attachment to the cell envelope is a weak point in the whole structure: according to Pierucci and Zuchowski [10], in fact, only one of the two DNA strands is attached to the envelope.

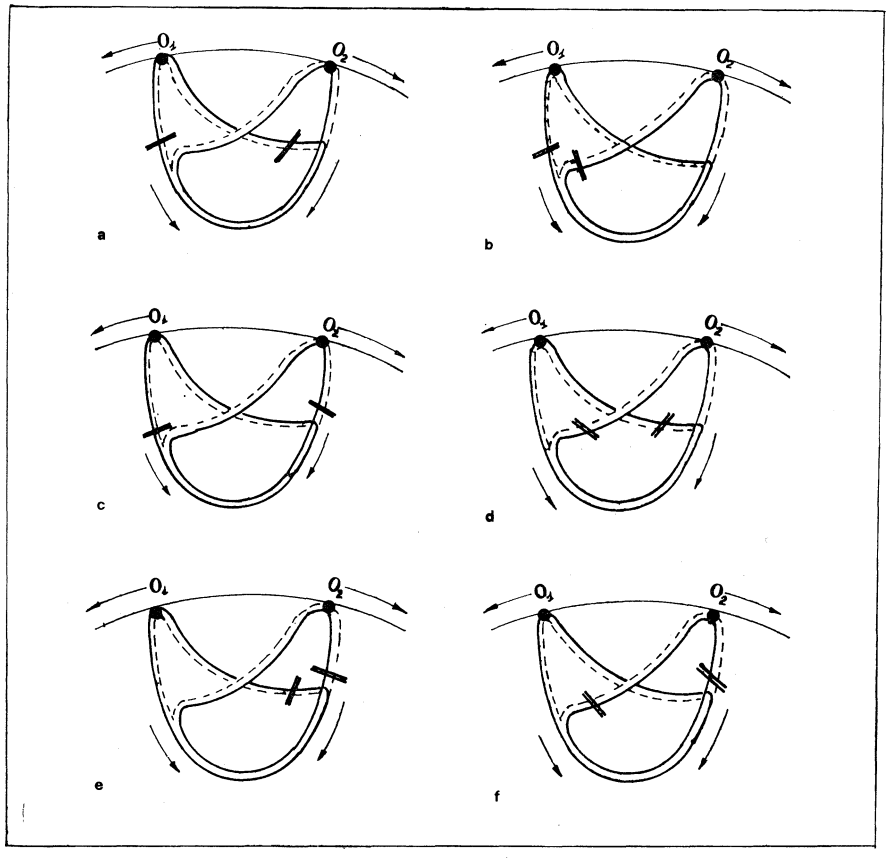

Fig. 4. – The six possible cases where two among the four already duplicated chromosome strands are broken.
a, f – only one of the two daughter cells is able to divide; b, c – the two breaks are not sufficient for the unloading of the mechanical tension; e, d – the daughter cells are both unable to divide.

As far as we know there are no examples of mechanical breakage of covalent bonds in biological processes. However, the detachment from the cell envelope may not be the product of a cut in the DNA strands, but only their removal from a region (presumably proteic) of the envelope interacting with the chromosome through secondary bonds.

These bonds are normally reversible, so one may wonder if the anchorage can at least occasionally reconstruct spontaneously in the daughter cell. It seems unlikely that this could happen in an appreciable fraction of the cells, because

of the precise quantitative ratios between viable and non viable cells, in the growth curves of Fig. 3.

Finally, it is to be shown that a cell whose chromosome is intact but detached from the wall is actually incapable of duplicating its DNA and of further dividing, even if protein synthesis has been going on at least for a certain time.

The assumption that detachment or cut of DNA under tension is effected by an enzyme is, at first sight, the most probable one. In fact, it is known that all cell processes are somehow mediated by enzymes. Even without hypothesizing a specific enzyme, one can think, for example, that a topoisomerase recognizes DNA under tension in the same way it recognizes DNA in a positive or negative torsion state [11]. The cut operated by such an enzyme would be irreversible in this case, because a sudden discharge of the mechanical tension would produce a definitive separation of the two ends of the molecule. However, it is not clear why the action of the enzyme should be effective only at the origin and not in the proximity of the replicating forks (this last case being left out on the ground of the previous considerations).

We have no elements to decide between the two alternative assumptions of a mechanical detachment or of a cut effected by an enzyme.

However, it seems difficult to reject the conclusion that the irreversible damage, which makes one of the two daughter cells incapable of forming colonies on agar, is somehow induced by a sudden yielding of the chromosome structure, discharging the mechanical tension generated by the block of the replicating forks under the action of NAL.

Some of the growth curves on which the present discussion is based were obtained by doctors C. Bacci and O. M. Quaranta.

This research was supported by " Consiglio Nazionale delle Ricerche (C.N.R.) – Comitato per la Fisica ", and carried out as part of the activity of the " Gruppo di Biofisica " of the Physical Institute of the University of Rome, associated with the " Centro di Studio Acidi Nucleici " of C.N.R.

References

[1] Ageno M., Bacci C. and Quaranta O. M. (1980) – *Cell cycle regulation in* Escherichia coli *I*, « Rend. Acc. Naz. Lincei », *68*, 139.

[2] Jacob F., Brenner S. and Cuzin F. (1963) – *On the regulation of* DNA *replication in bacteria*, « Cold Spring Harbor Symp. Quant. Biol. », *28*, 239.

[3] Thilo L. and Vielmetter W. (1976) – *Independence of deoxyribonucleic acid replication and initiation from membrane fluidity and the supply of unsaturated fatty acids in* Escherichia coli, « J. Bacteriol. », *128*, 130.

[4] Pardee A. B., Wu P. C. and Zusman D. R. (1973) – *Bacterial division and the cell envelope*, in Leive L. ed *Bacterial Membranes and Wall*, Dekker, New York, pp. 375–412.

Helmstetter C. E. (1974) – *Initiation of chromosome replication in* Escherichia coli *II. Analysis of control mechanism*, « J. Mol. Biol. », *84*, 21.

[5] STONINGTON G. O. and PETTIJOHN D. E. (1971) – *The folded genome of* Escherichia coli *isolated in a protein*–DNA–RNA *complex*, « Proc. Natn. Acad. Sci. USA. », *68*, 6.
WORCEL A. and BURGI E. (1972) – *On the structure of the folded chromosome of* Escherichia coli, « J. Mol. Biol. », *71*, 127.
PETTIJOHN D. E. and HECT R. (1973) – RNA–*molecules bound to the folded bacterial genome stabilize* DNA *folds and segregate domains of supercoiling*, « Cold Spring Harbor Symp. Quant. Biol. », *38*, 31.
WORCEL A., BURGI E., ROBINSON J. and CARLSON C. L. (1973) – *Studies on the folded chromosome of* Escherichia coli, « Cold Spring Harbor Symp. Quant. Biol. », *38*, 43.
DELIUS H. and WORCEL A. (1973) – *Electron microscopic study on the folded chromosome of* Escherichia coli, « Cold Spring Harbor Symp. Quant. Biol. », *38*, 53.
DELIUS H. and WORCEL A. (1974) – *Electron microscopic visualization of the folded chromosome of* Escherichia coli, « J. Mol. Biol. », *82*, 107.

[6] HELMSTETTER C. E., PIETRUCCI O., WEINBERGER M., HOLMES M. and TANG M. S. (1979) – *Control of cell division in* Escherichia coli, in: SOKATCH J. R. and ORNSTON L. N. eds. *The Bacteria : vol. VII. Mechanisms of Adaptation*, Academic Press, New York, pp. 517–579.

[7] COZZARLI N.R. (1980) – DNA *gyrase and the supercoling of* DNA, « Science », *207*, 953.

[8] RAMAREDDY G. and REITER H. (1969) – *Specific loss of the newly replicated deoxyribonucleic acid in nalidixic acid–treated* Bacillus subtilis *168*, « J. Bacteriol. », *100*, 724.

[9] GOSS W. A., DEITZ W. H. and COOK T. M. (1964) – *Mechanism of action of nalidixic acid on* Escherichia coli, « J. Bacteriol. », *88*, 1112.
GOSS W. A., DEITZ W. H. and COOK T. M. (1965) – *Mechanism of action of nalidixic acid on* Escherichia coli *II. Inhibition of deoxyribonucleic acid synthesis*, « J. Bacteriol. », *89*, 1068.

[10] PIERUCCI O. and ZUCHOWSKI C. (1973) – *Non–random segregation of* DNA *strands*, J. Mol. Biol. », *80*, 477.

[11] Si veda ad esempio: ALBERTS B. ed. *Mechanistic Studies of* DNA *Replication and Genetic Recombination*, Academic Press, New York, 1980. In particolare: « IX. DNA Topoisomerases », pp. 769–837.