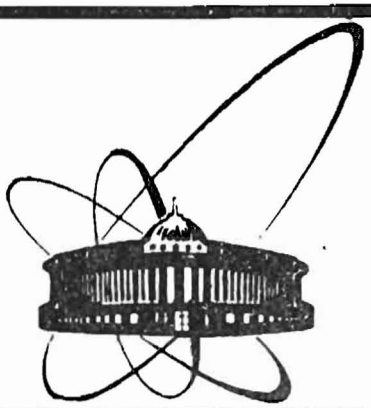

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THE INVESTIGATION OF SOS-RESPONSE OF ESCHERICHIA COLI AFTER γ-IRRADIATION BY MEANS OF SOS-CHROMOTEST

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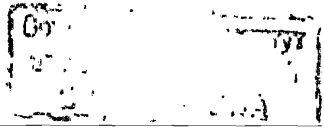
Introduction

DNA damaging agents in E.coli are known to induce an SOS-reaction during which a large number of biochemical events are realized. There is a single system that controls the expression of genes involved in the SOS-response. The SOS-system is connected with recA and lexA genes. The most important cell properties are under control of this system - DNA repair, mutagenesis, cell division, prophage induction of lysogens, etc.

So a study of SOS-system induction is essential for clearing up the mechanism of action of different DNA-damaging agents on bacterial cells. To this aim the bacterial strain E.coli PQ37 turns out to be a convenient object.

The strain was constructed by fusion of sulA and lacZ operons (sulA::lacZ). So, the expression of sulA gene controlled by lexA and recA proteins (SOS-system) can be detected by monitoring the level of ß-galactosidase. Quillardet et al. (Quillardet et al., 1982) proposed a SOS-chromotest technique for quantitative measurement of the action of DNA-damaging agents using this bacterial strain. The SOS-chromotest is based on simultaneous colorimetric assay of ß-galactosidase and alkaline phosphatase activities. The SOS-chromotest has been already used (Ohta et



al., 1984; Kozubek et al., 1987). Nevertheless the kinetics of SOS-system induction was far from being known. The knowledge of this kinetics is essential both for understanding the SOS-system induction itself and for correct estimation of mutagenic influence of different agents. We devoted the present paper to the kinetics of SOS-system induction by γ-irradiation in E.coli PQ37 using the SOS-chromotest.

Material and Methods

Bacterial strain. In all the experiments we used Escherichia coli K-12, the strain PQ37, with a genotype described in (Huisman and D Ari, 1981).

γ-irradiation. The stationary-phase culture (18 hours) was diluted 1:10 in a fresh LA-medium and shaken for 2 hours at 37°C to the cell density of about 10^8 cells/ml (Quillardet et al., 1982). Then the cells were diluted 10-fold in a fresh LA-medium and distributed into glass test tubes. After γ-irradiation by different doses the cells were incubated for an additional time at 37°C with shaking. In different time intervals the samples were taken for the assay of ß-galactosidase and alkaline phosphatase.

ß-galactosidase assay. The assay was described by (Quillardet et al., 1982) except for a little modification. Cell membranes were disrupted with 0,1% SDS (sodium disulfate) in the Z-buffer during 10 min at room temperature. The enzyme reaction was started by adding ONPG (4μg/ml in the phosphate buffer, pH=7,0) and stopped by adding 1 M Na_2CO_3. The colour reaction was measured spectrophotometrically at 420 nm by a Specol-11 spectrometer (Zeiss, DDR). A comparison was made with the reference sample of cells which were nor irradiated neither incubated. The sample was kept on ice.

Alkaline phosphatase assay. The assay was performed as described by (Quillardet at al., 1982) except for T-buffer suplemented with 0,1% SDS to disrupt cell membranes.

Protein determination. The contents of protein in cell suspension was measured by the Lowry method (Lowry et al.,1951).

Determination of cell survival. The cell survival was determined by the standard method, i.e. by counting a number of macrocolonies on the nutrient agar. The radiosensitivity was calculated as the slope of exponential dose-response dependences of stationary-phase cultures or as the final slope of shouldered cell survival curves of an exponential-phase cultures incubated before irradiation for two hours in fresh LA-medium. Irradiation was performed in the M9-buffer.

Calculation of the induction factor. The induction factor is the ratio of the normed ß-galctosidase activity to the normed alkaline phosphatase one:

$$IF(D) = \frac{g(D)/g(0)}{p(D)/p(0)}, \qquad (1)$$

where g(D) and g(0) are the ß-galactosidase activity in γ-irradiated cells and intact cells, respectively; p(D) and p(0) are the same for alkaline phosphatase. The derivative of IF(D) for the zero dose is called SOSIP (SOS-induction potency).

The determination of parameters entering into different equations was performed by the minimization of the sum of deviations squared of theoretical and experimental values.

Results and discussion

1. The growth curve of E.coli K-12, strain PQ37.

Intact cells were cultivated in a LA-medium at 37°C with

shaking. The growth rate was examined for 0-180 min (Fig.1.). The growth curve is exponential:

$$N(t) = N_o e^{\beta_o . t} ; \qquad (2)$$

where N(t) and N_o are the numbers of cells at moments t and o, respectively. The slope of the semilogarithmic graph corresponds to the generation time T_o=46 min and consequently β_o= =ln2/T_o=0,0152 min^{-1}.

2. Survival after γ-irradiation. This dependence was determined for stationary - phase culture and exponential -phase one (Fig.2.). The survival curve of the stationary - phase culture was exponential up to 630 Gy. The sensitivity (Do^{-1}) determined by the slope of the survival curve at the semilogarithmic scale was Do^{-1}=0,014 Gy^{-1}, i.e. Do=72 Gy. The shouldered survival curves of the exponentially growing cells could be characterized by the same sensitivity (the same final slope). The extrapolation number (n) was equal to 3. So the cell survival after γ-irradiation with dose D could be represented by a function

$$S(D) = 1 - (1 - e^{-D/Do})^n , \qquad (3)$$

where Do=72 Gy and n=3.

3. The growth curves of cell cultures after γ-irradiation. The number of viable cells was measured in different periods (t) of cultivation. The number of cells at a dose D and moment t was normalized to the number of cells at D=0 Gy, t=0 min. The averaged data of five experiments are plotted in Fig.1-3. The data show that no delay in the cell growth was observed for the doses used in our experiments (see Fig.3). Such a phenomenon can

Fig.1. The relative number of E.coli PQ37 cells incubated at 37°C with shaking t minutes after γ-irradiation with doses 0 Gy (x), 20 Gy (+), 40 Gy (▲), 80 Gy (●), 160 Gy (▼).

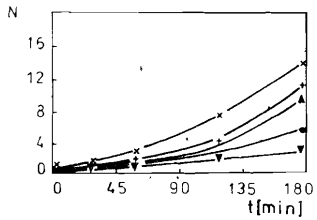


Fig.2. The survival curve of γ-irradiated E.coli PQ37 cells: stationary-phase cells (a), exponentially growing cells (b); S is the cell survival (relative number), D is the γ-irradiation dose in Gy.

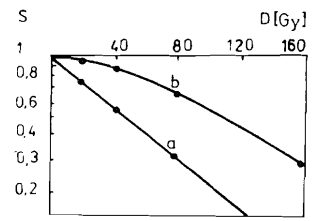
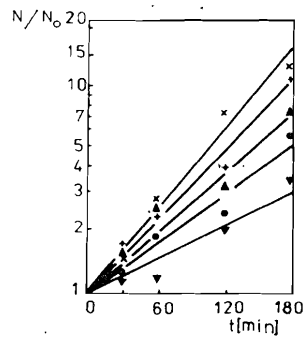


Fig.3. The relative amount of E.coli PQ37 cells irradiated with a γ-irradiation dose D (notation as in Fig.1) and incubated for t min (a semilogarithmic plot).



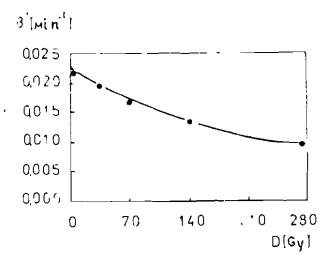


Fig.4. Slopes of the curves in Fig.3 ($\beta' = 1/T_o$) plotted against the γ-irradiation dose.

be connected with better effeciency of the repair processes in irradiated cells incubated under aerated conditions. This could compensate a delay in the growth of cells at small doses. The dependence of the quantity $\beta_D=1/T_d(D)$ on the γ-irradiation dose is given in Fig.4. It can be described by the function

$$\beta_D=\beta_o \exp(-\xi * D),$$

where $\beta_o=0{,}0152\ min^{-1}$, $\xi=0{,}0037$, and β_D is the slope of the growth curve at a dose D. Using equations (2) and (3) we obtain N(D,t) for different doses and different incubation times:

$$N(D,t) = S(D) * \exp(\beta_D * t). \qquad (4)$$

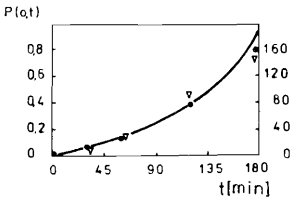
The curves generated by this equation are in good agreement with the experimental data.

4. Synthesis of alkaline phosphatase and protein in intact cells E.coli PQ37. Fig.5 represents the data on the alkaline phosphatase activity in the intact E.coli PQ37 suspension incubated for various times with shaking at 37°C. The experimental data are plotted as points while the theoretical curve corresponds to the equation

$$p(0,t)=\Omega * [\exp(\beta_o . t) - 1] , \qquad (5)$$

where $\Omega=0{,}068$. The formula was derived on the assumption that the rate of the alkaline phosphatase synthesis is constant and so the increment of the enzyme activity dp is proportional to the number of cells in the suspension at the time t, i.e. $dp=\exp(\beta_o . t) * dt$. The coefficient Ω depends on several

Fig.5. The activity of alkaline phosphatase p(0,t) (left scale) and of the protein amout (right scale) in a suspension of intact cells E.coli PQ37 aerated for t min at 37°C. Theoretical curve was calculated according to Eq.5.



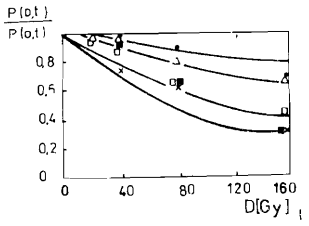


Fig.6. The relative activity of alkaline phosphatase in a suspension of cells E.coli PQ37 p(D,t)/p(0,t) as a function of the γ-irradiation dose D after aeration for t=30 min (•), 60 min (△), 120 min (□); the same scale for protein t=120 min (×), 180 min (■). Theoretical curves were calculated according to Eq.6.

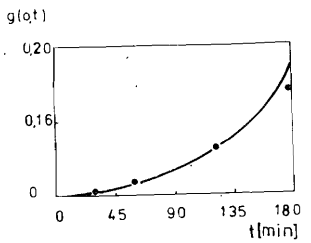
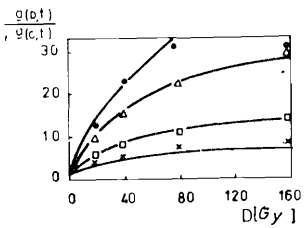


Fig.7. The β-galactosidase activity g(0,t) of intact cells E.coli PQ37 during aeration for t minutes. Theoretical curve was calculated according to Eq.5.

Fig.8. The relative activity of β-galactosidase in a suspension of cells E.coli PQ37 g(D,t)/g(0,t) as a function of the γ-irradiation dose D after aeration for t= =30 min (•), 60 min (△), 120 min (□), and 180 min (x). Theoretical curves were calculated according to Eq.5 and Eq.7.



biochemical parameters: the rate of alkaline phosphatase synthesis, the generation time, and the initial level of alkaline phosphatase.

The same regularities were also observed in the case of general protein contents in the cell cultures (the right scale in Fig.5). So the phosphatase synthesis occurs in parallel to the protein synthesis in the exponentially growing intact cells. It should be mentioned that there is no strict correlation between the measured activity of alkaline phosphatase and the density of cells in the sample of suspension. Such a correlation however, arises for greater time periods as it can be seen from Eq.5.

5.Alkaline phosphatase activity in γ-irradiated E.coli PQ37. The alkaline phosphatase activity was measured in different intervals of incubation of the γ-irradiated cells. The experimentally obtained ratios $p(D,t)/p(0,t)$ are given as the points in Fig.6. It can be seen that these ratios decrease as the γ-irradiation dose increases. Subsequent incubation of irradiated cells enhances this tendency.

The equation

$$p(D,t)=\frac{[S/\beta_o]*[\exp(\beta_p.t)-1] + [1-S]*t}{[1/\beta_o]*[\exp(\beta_o.t)-1]} \qquad (6)$$

satisfactorily describes the obtained dependence of the alkaline phosphatase activity on the γ-irradiation dose and on the incubation time. The first term describes the phosphatase activity of dividing cells and the second one gives the enzyme activity of γ-damaged cells that are unable to form colonies. Good agreement of Eq.6 with the experimental data allows us to formulate the following hypothesis. In lethally

damaged cells the alkaline phosphatase synthesis is not stopped; its rate remains constant during the whole experiment (180 min). It means that the phosphatase synthesis is not markedly changed in the γ-irradiation damaged cells at both the transcription and translation levels

6.β-galactosidase synthesis in intact E.coli PQ37. Fig.7 represents the experimental data on the β-galactosidase activity in the suspension of intact cells incubated with shaking at 37°C. The inducible enzyme activity of exponentially growing cells (180 min) increases in the way similar to that of the constitutive enzyme activity of alkaline phosphatase. Therefore, the time dependence can be described by the same equation (Eq.5). The average rate of the enzyme synthesis could be connected with some constant probability of SOS-induction (per cell per time unit).

7.The dependence of β-galactosidase activity on γ-irradiation dose and incubation time. The experimentally obtained ratios $g(D,t)/g(0,t)$, where $g(D,t)$ is the activity of the enzyme at dose D and time t, are given as the points in Fig.8. The data show that the enzyme activity is sharply increased with an increase of the γ-irradiation dose. Then the dependence is saturated at approximately 100 Gy. Fig.8 also shows that the normed β-galactosidase activity decreases during the incubation.

The obtained dependence allows two interpretations. In the first one the SOS-system induction efficiency depends on a number of damages in cells, i.e. the higher is the dose, the greater is the number of damages in cells, and the greater is the SOS-response. In the second interpretation one assumes that the enzyme activity increases proportionally to the number of damaged cells.

It seems imposible at present time to make a comparative analysis of the efficiency of SOS-system induction in a separate cell. Therefore, we shall use the second interpretation and show that it allows a good description of the experimental data.

Let $\alpha.D$ be the yield of DNA-damages inducing the SOS-system. Then $\exp(-\alpha.D)$ will give the number of undamaged cells, and $1-\exp(-\alpha.D)$ will represent the number of damaged cells. The ß-galactosidase activity will be proportional to the last term and will have a plateau at some dose.

Now we shall discuss the dependence of the ß-galactosidase activity on the incubation time of γ-irradiated cells. This dependence is connected to the repair processes that occur during incubation and have a stochastic nature. If δ is the probability of the cell reparation per time unit, one can expect that $\exp(-\delta.t)$ will give the fraction of cells that are able to accomplish the inducible repair during time interval [0,t]. So the dependence of the ß-galactosidase activity on the γ-radiation dose and incubation time will have a form

$$g(D,t)=a.[1-\exp(-\alpha.D)]*[1-\exp(-\delta.t)], \qquad (7)$$

where a is the proportionality constant. The theoretical curve agrees with experimental data points for the following values of the parameters: a=0,1783; α=0,021 Gy^{-1}; δ=0,005 min_{-1}.

These data permit us to determine the dose which causes on an average one SOS-inducing injury per cell - D^{SOS}= 48 Gy. The SOS-system is switched off approximately 200 min after γ-irradiation.

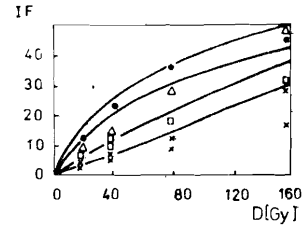


Fig.9. The value of the induction factor (IF) calculated according to Eq.1 as a function of the γ-irradiation dose for E.coli PQ37 cells incubated for different periods of time: 30 min (•), 60 min (△), 120 min (□), 180 min (×) (the data are normalized to phosphatase and protein). Theoretical curves were calculated according to Eqs.5-7.

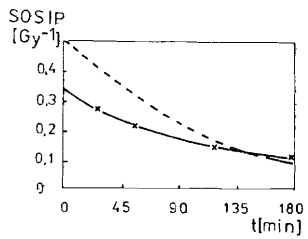


Fig.10. The SOSIP values plotted versus the incubation time (solid line), and the theoretical curve generated by Eqs.6 and 7 (dashed line).

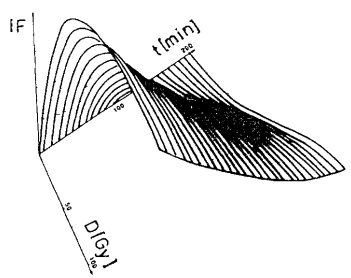


Fig.11. The induction factor as a function of the γ-irradiation dose and of the aeration time at 37°C in three dimensional plot. Wires method with hidden line subroutine was used for calculation of the graph.

8. Determination of induction factor

Quillardet et al. (Quillardet at al., 1982) have shown that one can judge about the ability of various mutagenic agents to induce the SOS-system in the E.coli PQ37 cells by the value of

the induction factor IF. This factor is calculated by relative values of the ß-galactosidase and alkaline phosphatase activity in a suspension of cells incubated for 120 min (Eq.1). In our work we have obtained values of IF(D,t) for different periods of incubation after r-irradiation. To calculate the induction factor, we took the values of the enzyme activity for the corresponding time. Fig.9 shows the experimental points and the theoretical curves generated by equations (6) and (7). The theoretical and experimental IF values agree at medium and high r-doses in different time intervals; agreement is worse at small doses. It should be noted that the fluctuation of IF values is less than that of the enzyme activity values, because they are statistically correlated.

9. Induction potency of SOS-system (SOSIP)

The induction potency of the SOS-system is characterized by the slope of the IF(D) curve. In our work we can judge about the state of the SOS-system in different periods of incubation. Fig. 10 shows the values of SOSIP obtained experimentally (solid line) and calculated by equations (6) and (7) (dashed line). As seen, the values of SOSIP decrease with the cell incubation time. Comparison of the experimental and theoretical curves reveals better agreement at a longer incubation time.

Discusion and Conclusions

The data obtained allow the following conclusions. The growth of E.coli PQ37 cells in a LA medium aerated for 180 min. at 37°C is described by an exponential curve. The cell generation time is 46 min under these conditions. Sensitivity of the cells to r-irradiation both in the stationary and exponential growth

phases is characterized by the quantity D_o=72 Gy. If the cells were additionally incubated before irradiation, the exponential survival curve acquires a shoulder with the extrapolation number equal to 3.

It follows from the experimental data that constitutive alkaline phosphatase and all the protein are synthesized at an approximately constant rate in intact and irradiated cells. Besides, it is shown that no strict correlation is observed between the amount of the enzyme and protein and the number of cells.

The dependence of the activity of irradiated cells on the incubation time is well described by equation (6) only if all cells, including the lethally damaged ones, are taken into account. This important observation indicated that synthesis of constitutive alkaline phosphatase continues for some time after the cell is lethally damaged by irradiation. The synthesis is assumed to take place both at the transcription and translation levels.

The activity of inducible ß-galactosidase sharply increases at incubation after r-irradiation. The detected increase in the activity of this enzyme during incubation of intact cells is of great interest, since it indicates that the SOS-system can be switched on in exponentially growing cells.

The increase in the activity of ß-galactosidase after r-irradiation is shown to strictly depend on the dose and consequently, on the number of damages or damaged cells. This dependence indicates that r-damages are a substrate for triggering of the SOS-system in E.coli cells.

It follows from the experimental data and calculations that the yield of damages triggering the SOS-system is 0,021 Gy^{-1} per cell, and the time of the SOS-system switching off is on the average 200 min.

The induction factor is a very important and quite suitable quantity to be used in the comparative analysis of mutagenic action of different agents. It can be a measure of such action but its calculation shows some weak points worth discussing. The induction factor is the quotient of relative activities of the inducible enzyme $g(D,t)/g(0,t)$ and the constitutive enzyme $p(D,t)/p(0,t)$. During incubation the activity of these enzymes changes differently for different reasons. The constitutive enzyme activity $p(0,t)$ and $p(D,t)$ is mainly associated with the number of cells; living cells and all cells including those lethally damaged, respectively. The case of the inducible enzyme ß-galactosidase is more complicated. In intact cells its activity exponentially grows (Fig.7) in accordance with the exponential increase in the number of cells (Fig.1). This dependence is possible on the assumption that the probability of the spontaneous stochastic triggering of the SOS-system in those cells in the experimental incubation period is constant for a cell per unit time and does not depend on time. Consequently, the value of $g(0,t)$ is also associated with the exponentially changing number of cells.

To calculate IF, $g(D,t)$ is the most essential quantity. Note that the ß-galactosidase activity depends on the number of damaged cells at the moment t=0 rather than on the number of cells at t=120 min, i.e. it is related to the number of cells at the initial moment of time. After r-irradiation with sufficient

dose most cells suffer damages which inhibit their division. Moreover, the number of non-lethally damaged cells remains at the same level, untill the SOS-system is switched off (200 min). Therefore, it is reasonable to measure the ß-galactosidase activity of suspension with intact cells before the cells have divided, i.e. at a moment τ shorter than is the doubling time. The most suitable value of τ is the one that shows, on the one hand, the number of cells during irradiation and, on the other hand, the triggering of the SOS-system in them. This time must be positive and less than the cell generation time T_o. For example, if T_o=46 min (as in our case), the τ can be taken equal to 30 min. The correction factor t/τ must be introduced in the final calculation of IF. We are of the opinion that this yields a more accurate IF for the comparative analysis of action of different mutagens. The proposed correction is especially significant for dealing with mutagens whose presence inhibits growth of cells. In this case the value of IF will be a more reliable indication of the sos-system induction.

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Козубек С. и др.
E19-88-868

Исследование SOS-ответа клеток E.coli после γ-облучения методом SOS-хромотеста

Изучена кинетика индуцирования γ-облучением SOS-системы у бактерий E.coli PQ37 методом SOS-хромотеста. Показано, что синтез конститутивного белка — щелочной фосфатазы продолжается после репродуктивной гибели γ-облученных клеток. В экспоненциально растущих клетках E.coli PQ37 спонтанный запуск SOS-системы, вероятность которого в расчете на одну клетку в единицу времени постоянна, не зависит от времени. Установлено, что выход повреждений, запускающих SOS-систему, составляет 0,021 $Гр^{-1}$ на геном, время выключени SOS-системы равно в среднем ~200 мин. Для оценки индукции SOS-системы по методу Килларде и др. предложено вносить коррекцию в вычисление фактора индукции.

Работа выполнена в Лаборатории ядерных проблем ОИЯИ.

Препринт Объединенного института ядерных исследований. Дубна 1988

Kozubek S. et al.
E19-88-868

The Investigation of SOS-Response of Escherichia coli after γ-Irradiation by Means of SOS-Chromotest

The kinetics of the E.coli PQ37 SOS-system induction by γ-radiation has been studied by the SOS-chromotest technique. The experimental data are consistent with the following hypotheses. The production of DNA damages inducing the SOS-system is 0,021 Gy^{-1} per genome. The SOS-system is switched off approximately 200 min after γ-irradiation. The spontaneous triggering of the SOS-system is induced in the exponentially growing cells. The probability of its induction is independent of time up to 180 min of incubation. The synthesis of constitutive alkaline phosphatase proceeds for some time in the cells that suffered lethal damages from γ-irradiation. A correction has been proposed for the calculation of the induction factor.

The investigation has been performed at the Laboratory of Nuclear Problems, JINR.

Preprint of the Joint Institute for Nuclear Research. Dubna 1988