Effect of Potassium Nitrate on Photoreactivation of *Escherichia coli* Cells

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The increase of survival of ultraviolet irradiated *Escherichia coli* cells, due to the splitting of thymine dimers to monomers by photoreactivation, is inhibited by potassium nitrate. The possible mechanisms of the inhibition are discussed.

Photoreactivation is the repair of ultraviolet radiation damage in a biological system with light of wavelength longer than that of the damaging radiation (Jagger [1]). A wide variety of ultraviolet induced effects may be reversed by photoreactivation, including killing, mutations, division delay, block in the DNA synthesis, etc. Such photoreactivation effects generally involve the photoenzymatic splitting of cyclobutane-type pyrimidine dimers in the DNA (Rupert [2]).

The effect of potassium nitrate was examined on the photoreactivation of colony forming ability of ultraviolet irradiated *Escherichia coli* cells and on *in vivo* monomerization of ultraviolet induced thymine dimers in the bacterial cell's own DNA during photoreactivation.

Materials and Methods

Bacteria

The bacterial strains used were *Escherichia coli* B_{s-1}, and *Escherichia coli* 15 T^- 555-7 thy^-, arg^-, trp^-, met^-, the kind gift from Dr. D. Billen. *E. coli* B_{s-1} cells were grown in a synthetic glucose medium (Sedliaková *et al.* [3]). *E. coli* 15 T^- 555-7 cells were grown in the same glucose medium containing 14 μg tryptophan, 30 μg methionine, 38 μg arginine and 2 μg thymine plus radioactive thymine-2-^{14}C (0.5 μCi/ml, specific activity 53 mCi/mM). The complete medium was inoculated and then incubated in a reciprocal shaker at 37 °C. The overnight culture was diluted into pre-warmed fresh medium and grown until cells were in stationary phase. The titer of *E. coli* B_{s-1} cells in stationary phase was $3 \cdot 10^9$ colony forming units (CFU) per ml and the titer of *E. coli* 15 T^- 555-7 was $4 \cdot 10^8$ CFU/ml. Cells were harvested by membrane filtration, washed and resuspended in an equal volume of mineral salts buffer (MSB) composed of 7 g $Na_2HPO_4 \cdot 7\,H_2O$, 3 g KH_2PO_4 and 4 g NaCl, dissolved in 1000 ml H_2O, to which 4 ml of 0.5 M $MgSO_4$ solution was added after autoclaving.

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Ultraviolet irradiation

The ultraviolet source was the Philips TUV 15 W low-pressure mercury-vapor germicidal lamp, emitting mainly the wavelength 253.7 nm. At this wavelenght the dose rate was 6.3 $erg \cdot mm^{-2} \cdot sec^{-1}$. For applying small doses, the dose rate was reduced to 2.5 $erg \cdot mm^{-2} \cdot sec^{-1}$. Dose rates were determined with a Latarjet N° 90 dose-rate-meter. Cell suspensions were irradiated in open Petri dishes in a 1 – 2 mm thick layer with rapid stirring. The cells were starved in MSB for 24 hours before each experiment. All manipulations with irradiated cells besides photoreactivation were carried out under yellow light from NARVA Na-E spectral lamps which do not emit at wavelengths below 568.8 nm.

Potassium nitrate treatment

Potassium nitrate was dissolved at 2 M concentration in the MSB. Further dilutions were made from this solution and appropriate amounts were added to the samples immediately after ultraviolet irradiation. Cells were kept in these solutions at 20 °C between irradiation and photoreactivation and also during photoreactivation.

Photoreactivation

The sources for photoreactivating light were 2 closely spaced Tesla Tovos RVK 250 W high pressure mercury lamps. A 20 °C filter bath, containing 15% $CoSO_4 \cdot 7\,H_2O$ and 17.5% $CuSO_4 \cdot 5\,H_2O$ in

water was placed in front of the lamps. Samples in glass reaction tubes were immersed in this bath. The path length of photoreactivating light in filter bath was 2 cm, so that 92% of the light was between 320 and 450 nm. Cells to be photoreactivated were at a concentration not exceeding $5 \cdot 10^5$/ml; the cell concentration was up to $4 \cdot 10^8$/ml in experiments for the thymine dimer determination.

Determination of colony forming ability

Suitably diluted samples of cultures were plated on supplemented minimal salts medium agar plates. Plates were incubated at 37 °C in the dark and the colonies counted next day.

Photoproduct analysis

Thymine dimers were determined in formic acid hydrolysates of high-molecular (cold trichloroacetic acid-insoluble fraction) DNA by paper radiochromatography. For details see Sedliaková *et al.* [4].

Compounds

Most of the compounds used in this study was obtained from Lachema, Brno. Radioactive thymine was purchased from the Institute for Research and Production of Radioisotopes, Prague.

Results and Discussion

The rate of photoreactivation for irradiated *E. coli* B_{s-1} cells in the presence and in the absence of KNO_3 is shown in Fig. 1. Survival increase is slower when KNO_3 is present (curves 2, 3, 4) than when it is absent (curve 1). However, while not shown in Fig. 1, all curves attain the same final level. Inhibition effect of KNO_3 increases with increasing KNO_3 concentration through the range studied. At a constant concentration, the rate of photoreactivation is slower when the light intensity is reduced (curves 4, 5).

There is no effect of 0.5 M KNO_3 on the unirradiated and irradiated samples in the dark and

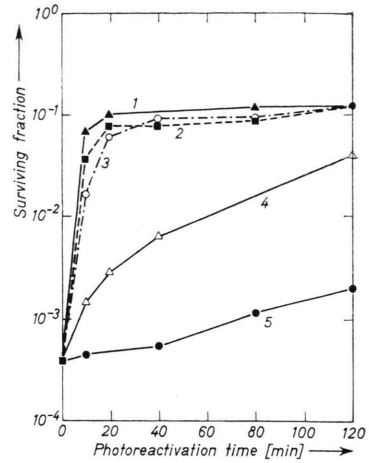

Fig. 1. Survival increase of *E. coli* B_{s-1} cells as a function of photoreactivating time. Cells were irradiated with 14 erg·mm^{-2}. Immediately thereafter, KNO_3 was added to four samples at a final concentration of 0.125 M (curve 2), 0.25 M (curve 3) and 0.5 M (curves 4 and 5). Corresponding amount of MSB was added to control sample (curve 1). Cells were then kept 30 min at 20 °C in the dark; after this period they were exposed to photoreactivating light from two RVK 250 W lamps, except one sample (curve 5) which was exposed to light from only one lamp.

photoreactivating light has no effect on the unirradiated samples in the presence and in the absence of KNO_3 (Table I).

The same type of experiments was carried out with the radiation resistant *E. coli* 15 T$^-$ 555-7 strain. Fig. 2 shows that photoreactivation of cells results in a large reduction of the thymine dimer concentration. Furthermore, the reduction is greater in the absence of KNO_3 than in its presence. Incubation of samples in the dark has no effect on the initial thymine dimer concentration.

The above results clearly indicate that KNO_3 inhibits the splitting of thymine dimers. Therefore, it must directly inhibit photoreactivation. Photoreactivation can be described by the following reaction scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[k_3]{\text{light}} E + P,$$

Table I. Effects of KNO_3 and the photoreactivating light on the titer of *E. coli* B_{s-1} cells. Unirradiated sample 1 was held in 0.5 M KNO_3 as well as irradiated (10 erg·mm^{-2}) sample 2. Sample 3 (unirradiated) was illuminated with photoreactivating light. Unirradiated sample 4 was illuminated with photoreactivating light in the presence of 0.5 M KNO_3.

Time [min]	Sample 1 [· 10^9 cells/ml]	Sample 2 [· 10^6 cells/ml]	Sample 3 [· 10^9 cells/ml]	Sample 4 [· 10^9 cells/ml]
0	2.1	2.2	2.8	2.9
40	2.4		1.8	2.6
80	2.0	2.5	2.7	
120				2.4

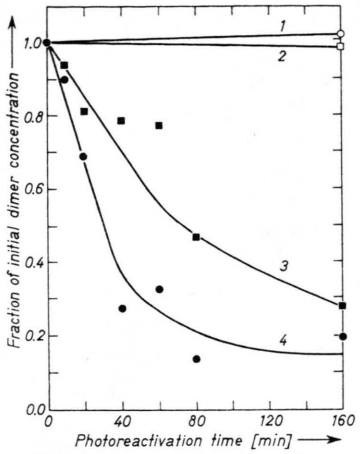

Fig. 2. The fraction of initial thymine dimer concentrations after various times of photoreactivation. *E. coli* 15 T^- 555-7 cells were irradiated with 1000 erg·mm^{-2}. After this dose, the initial fraction of radioactive thymine appearing in thymine dimers was 0.21%. Cells were then treated with KNO_3 and photoreactivated in the presence of KNO_3 (curve 3) and in its absence (curve 4). Curve 1, dark control in the presence of KNO_3; curve 2, dark control in the absence of KNO_3. Concentration of KNO_3 was 0.5 M.

where E is the photoreactivating enzyme, S is the substrate (a pyrimidine dimer in irradiated DNA), ES is the enzyme-substrate complex formed in the dark and P is the repaired product – pyrimidine monomers in DNA (Ruppert [2]). Thus, the inhibiting effect of KNO_3 could affect either the formation of ES complexes or the photolysis (light reaction) of existing complexes.

Harm *et al.* [5] have shown that the formation of ES complexes in *E. coli* B_{s-1} cells is inhibited by caffein; on the other hand, caffein does not interfere with the photolysis of existing ES complexes. They have observed decreased caffein inhibition of photoreactivation at low intensity of photoreactivating light. Rate constants k_1 for formation, and k_2 for dissociation of the ES complexes *in vitro* depend critically on the salt concentration; the photolysis constant k_3 is independent *in vitro* of ionic strength over a range producing large effects on the rate of complex formation (Harm *et al.* [5], Cook [6]). It is possible that KNO_3 increases ionic strength *in vivo*, so that formation of ES complexes is affected.

Illumination at low light intensity of ultraviolet-irradiated cells in the presence of KNO_3 would result in decreased inhibition of photoreactivation, when formation of ES complexes would be inhibited in the presence of KNO_3. This conclusion is based on the theory of Harm *et al.* [5]: "During such (continuous) illumination there exists a quasi-steady state of ES complexes, which is determined by the rate of complex formation *vs* the rate of photolysis. ... Complex formation in UV-sensitive system (such as B_{s-1} cells ...) is slow due to the low concentration of substrate molecules, so that in the quasi-steady state only a small fraction of the photoreactivating enzymes is in complexed form. Under these conditions a slower complex formation (in the presence of caffein) would roughly proportionally reduce the steady-state concentration. ... Illumination at very low light intensity should increase the steady-state concentration of ES in B_{s-1} cells, there by decreasing the inhibitory effect (of caffein) on the photoreactivation." However, our conclusion is in discrepancy with the results presented in Fig. 1 (curves 4, 5), thus inhibition of ES complex formation could be excluded. Nevertheless, definite conclusion can be made after direct determination of the reaction rate constants k_1, k_2 and k_3.

KNO_3 could affect the photolytic reaction step in the photoreactivation. Results recently obtained in the model systems may throw some light on this problem:

Pyrimidine dimers are split in the presence of indole derivatives in aqueous solutions by irradiation at wavelengths where only the latter absorb light (Hélène and Charlier [7]). Irradiation of pyrimidine bases in the presence of 3-indolylacetic acid causes a photoreduction of pyrimidines; this photoreduction is inhibited when KNO_3 is added to the reaction mixture (Reeve and Hopkins [8]). In the frozen state, the fluorescence of the indole derivatives is quenched by pyrimidines and pyrimidine dimers (Hélène and Charlier [7], Montenay-Garestier and Hélène [9]). Indole fluorescence is quenched also by a series of electron acceptors and the degree of this quenching is related to the electron affinity of the acceptor. KNO_3 is a very efficient electron acceptor and quencher of indole fluorescence (Steiner and Kirby [10]). Pyrimidines and pyrimidine dimers form charge-transfer intermolecular complexes with indole derivatives in fluid aqueous solution or in frozen state (Hélène and Charlier [7], Montenay-Garestier and Hélène [9] and Pieber *et al.* [11]). Tryptophan, an indole derivative, photosensitizes formation of various light and temperature sensitive radicals in the *cis-syn* thymine dimers in frozen aqueous solution at 77 °K; the first step in this

reaction is probably formation of an anion radical of the thymine dimer Balgavý [12].

Formation of radicals in thymine dimer, monomerization of dimers, photoreduction of pyrimidines and quenching of indole fluorescence can be ascribed to electron transfer either from the indole derivatives in the excited state to pyrimidine derivatives in the ground state or directly in the excited state of the intermolecular complex. KNO_3 is an efficient inhibitor of this reaction.

Enzymatic photoreactivation – an enzyme-mediated, light-dependent splitting of pyrimidine dimers – could be such an electron transfer reaction (Hélène and Charlier [13]). The photoreactivating enzyme forms (charge-transfer?) complexes with pyrimidine dimers in DNA. These complexes, but not the free enzyme (Muhammed [14], Setlow [15]), absorb photoreactivating light. An electron may be transferred to the dimer from the excited state of the enzyme-substrate complex. The dimer anion is then monomerized (split) in the free radical reaction. Potassium nitrate seems to inhibit electron transfer in this reaction. Such inhibition of electron transfer in the enzyme-substrate complex might cause inhibition of photoreactivation of ultraviolet-irradiated *Escherichia coli* cells in the presence of KNO_3.

However, further experiments will be needed to clarify the model of photoreactivation presented above.

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