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Effect of Visible Light on Progressive Dormancy of *Escherichia coli* Cells during the Survival Process in Natural Fresh Water

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Some effects of visible light on the survival of *Escherichia coli* in waters of the Butrón river were studied by comparing illuminated and nonilluminated systems. The following count methods were used: CFU on a selective medium (eosin-methylene blue agar), CFU on a medium of recuperation (Trypticase soy agar with yeast extract and glucose), number of metabolically active cells by reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan, and total number of *E. coli* cells as determined by the acridine orange direct-count method. In the illuminated systems, decreases in CFU of *E. coli* and in the number of metabolically active cells were observed. However, no decline of the total number of *E. coli* cells was observed. By count methods, different stages of progressive dormancy of *E. coli* cells were determined to exist in illuminated systems. Culturable and recoverable cells were defined as viable cells, and metabolically active cells and morphologically intact cells were defined as somnicells. Indirect activity measurements were also done by using [^{14}C]glucose. In illuminated systems, a decrease of glucose uptake by *E. coli* cells was observed throughout the experiments. The assimilated fraction of [^{14}C]glucose decreased faster than the respired fraction in illuminated systems. The percentage of respired [^{14}C]glucose ($^{14}CO_2$ production) with respect to the total glucose uptake increased throughout the experiments, and the percentage of assimilated glucose decreased. Therefore, the visible light was also responsible for an additional inhibition of biosynthetic processes.

Research on factors affecting survival of *Escherichia coli* in natural waters is of great interest due to the importance of this microorganism as an indicator of fecal pollution in natural waters. Among these factors, the following are of great importance: predation (4, 7, 18, 19), temperature (1, 3, 5, 10, 20), and light and other physical and chemical factors (11, 13, 21, 23, 25).

Light has been mentioned on several occasions (4, 8, 9, 12, 16, 17, 19) as a decisive regulatory factor of *E. coli* survival in natural waters. However, in this type of study, the parameter normally used to quantify the survival of *E. coli* is the evolution of the number of CFU. The effect of luminous radiation on *E. coli* survival in natural waters can be observed by comparing the numbers of CFU in illuminated and nonilluminated systems. In this way, the effect of light on the survival of *E. coli* in natural waters leads to a sharp decline in the number of CFU throughout the study period. This kind of experiment gives us little information on the influence which light exerts on *E. coli* cells in natural aquatic ecosystems.

Several authors (13, 21, 24) came to the conclusion that the number of enteric bacteria present in a natural aquatic ecosystem can be very much higher than the number of enteric bacteria detectable by plate count methods. These authors (21, 24) concluded that an important fraction of the enteric bacteria present in aquatic ecosystems maintain some type of metabolic activity. Moreover, Grimes et al. (13) underline the importance of this fraction of enteric bacteria (which is undetectable by plate count methods) due to the fact that they can maintain their infectiveness, which constitutes a health risk.

The term viable has been used to refer to bacterial counts in standard culture media. However, in order to avoid confusion due to the different counts obtained by using different culture media, Dawe and Penrose (6) use the term CFU, which is appropriate for every culture medium and which has been used by other authors. The term viable has been left as a theoretical concept synonymous with culturable.

Recently, Roszak and Colwell (24), working with *E. coli* and *Salmonella enteritidis* in aquatic systems, proposed the term somnicell to define those cells which were not culturable in standard culture media but which were detectable by direct-count techniques. Moreover, these authors proposed a series of stages of progressive dormancy for *E. coli* and *S. enteritidis* in long-term experiments (1 or 2 months) carried out in marine aquatic environments.

Bailey et al. (2) observed that visible light provokes a decrease in the active transport of radioactively labeled amino acids in natural bacterial populations from the marine medium. From the conclusions of these authors, it is logical to think that indirect activity measurements based on the addition of a determined, radioactively labeled substrate to the samples to be studied would allow us to observe the effect of visible light on the metabolic activity of a certain population of enteric bacteria in a natural aquatic medium.

Radioactively labeled substrates are frequently used in ecologic studies directed towards estimating bacterial heterotrophic activity in natural aquatic ecosystems. Moreover, the use of ^{14}C-labeled substrates, essentially using the technique proposed by Hobbie and Crawford (15), offered us the possibility of differentiating the assimilated and respired fractions from the ^{14}C-labeled substrate taken up. Wright and Burnison (26), by using this technique, defined respiration (or mineralization) as the production of $^{14}CO_2$ and assimilation as the substrate retained in the cell (that is, substrate taken up but not respired).

This paper describes the effect of visible light on *E. coli*

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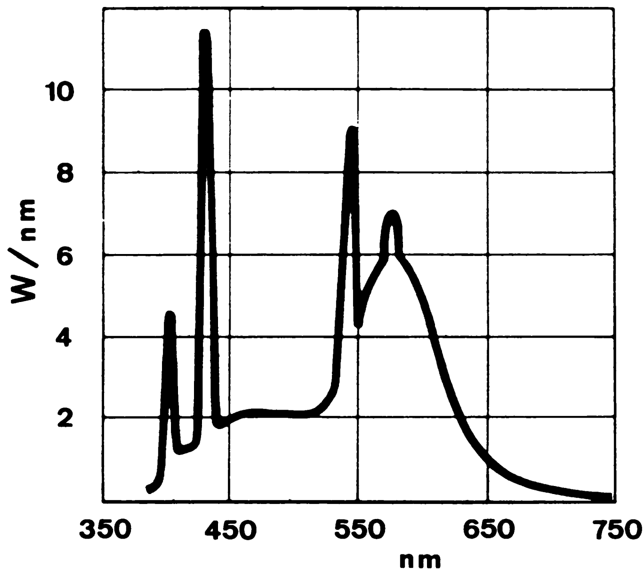


FIG. 1. Luminous spectrum of the lamps employed.

survival in waters of the Butrón river, taking into account its effect on the evolution of the number of CFU and on the metabolic activity (total uptake, assimilation, and respiration of [^{14}C]glucose) of *E. coli* cells.

MATERIALS AND METHODS

This study was carried out with water samples from the Butrón river in Spain. All samples were collected from the surface by using appropriate instruments.

An *E. coli* strain isolated from a river water sample and determined to be indole positive, methyl red positive, Voges-Proskauer negative, citrate negative, and Eijkman test positive was used throughout this study.

E. coli was grown in nutrient broth at 28°C for 8 h. Cells from the exponential phase were harvested by centrifugation (3,000 × *g* for 15 min) and washed three times with sterile saline solution (0.9%, wt/vol). The pellet was suspended in saline solution, inoculated in fresh nutrient broth, and incubated at 28°C for 18 h. The cells were harvested at stationary phase as described above. This final suspension was inoculated in the water samples at a final density of approximately 10^6 cells per ml.

All experiments were carried out in 2-liter flasks with 500 ml of sterile subsample. Sterile subsamples were obtained by filtering natural fresh water through 0.2-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). The incubation of inoculated subsamples was done at 20°C with centrifugation at 180 rpm in an orbital incubator with an illumination system consisting of eight Sylvania CW-ST 133 18-W lamps. The luminous spectrum of these lamps is shown in Fig. 1. We compared the evolution of the different parameters studied in illuminated and nonilluminated systems.

The CFU of *E. coli* were enumerated on a selective medium (Levine eosin-methylene blue [EMB] agar incubated at 37°C for 24 h) and on a medium of recuperation (Trypticase soy agar [BBL Microbiology Systems, Cockeysville, Md.] supplemented with 0.3% yeast extract and 0.5% glucose [TSY] [23]) incubated under the same conditions.

The total number of cells in the subsamples was estimated by the acridine orange direct-count (AODC) method of Hagström et al. (14). Subsamples were preserved in 2% formaldehyde (final concentration).

The cells with functional electron transport systems were considered cells that are able to metabolize nutrients, and we named them metabolically active cells. A direct activity count (DAC) method was used to determine these metabolically active cells, which would be able to reduce 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan in the presence of a nutrient, such as glucose, at a saturated concentration. The subsamples were incubated with 0.02% INT (final concentration) (Sigma Chemical Co., St. Louis, Mo.) and a saturated concentration of glucose for 40 min at 20°C in the dark and then preserved with 2% formaldehyde. The preserved subsamples were prepared for mounting and enumerated by the method of Newell (22).

The indirect activity measurements were done by using [U-^{14}C]glucose (250 mCi/mmol; Radiochemical Centre, Amersham, England) at a saturated concentration (100 μg of C per liter). Three 5-ml subsamples were analyzed for each uptake measurement, and they were incubated for 30 min in the dark at 20°C with shaking. After this incubation period, 40 μl of H_2SO_4 (2 N) was injected into the flasks and the subsamples were further incubated for 1 h to trap the $^{14}CO_2$ released by using a filter paper impregnated with β-phenetilamine (15). After both incubation periods, the entire volume of each subsample was filtered through 0.2-μm-pore-size membrane filters. The filters were rinsed three times with 5 ml of filtered water (0.2-μm-pore-size membrane filters), placed in Unisolve-1 (Hispanoland, Barcelona, Spain), and radioassayed by liquid scintillation counting. Quench curves were computed by the channel ratio method. Controls for abiotic absorption were prepared. Assimilated and respired fractions were defined by the method of Wright and Burnison (26).

RESULTS

Owing to the fact that the evolutions obtained were quite similar, only one experiment, which is representative of the seven carried out, is represented here.

Figure 2 shows an example of the results of the different cell counts carried out in both illuminated and nonilluminated systems during the survival studies. We observed that in the nonilluminated systems, the total number of cells (AODC method), the number of metabolically active cells (DAC method), the number of CFU on TSY, and the number of CFU on EMB did not undergo any important changes during the experiments. However, in the illuminated systems (Fig. 2), the number of CFU decreased four- and fivefold on TSY and EMB, respectively, during the experiment. In spite of this large decrease in the number of CFU during the incubation period, the total number of cells, determined by the AODC method, was constant during the experiments done in illuminated systems (Fig. 2). The number of metabolically active cells in illuminated systems as determined by the DAC method decreased during the incubation period, going from an initial 94.6 to 11.6% at the end of the period studied. The survival stages of the *E. coli* population throughout the experiments are summarized in Fig. 3, using the terminology proposed by Roszak and Colwell (24).

With regard to the results obtained in the uptake measurements of [^{14}C]glucose during the survival studies, we observed that in the nonilluminated systems, there was a slight increase in the total uptake throughout the experiment (Fig. 2). How-

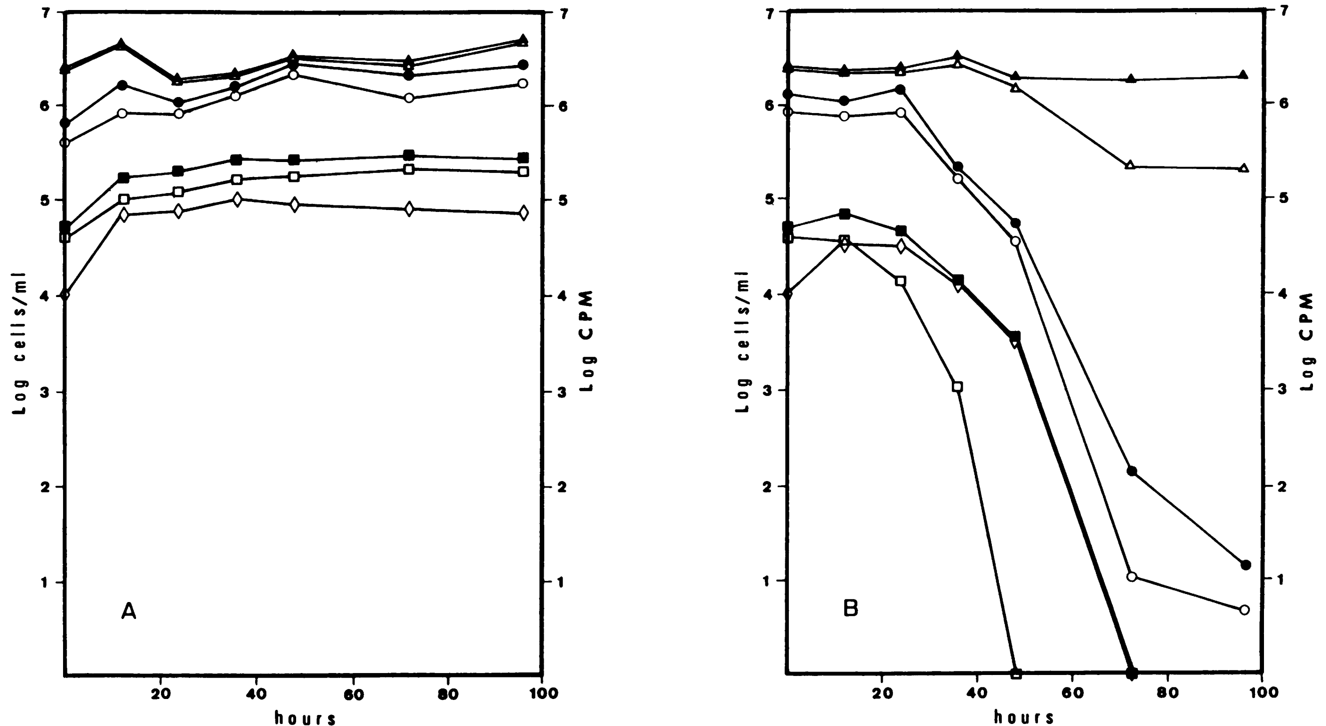


FIG. 2. Evolution of different counts and indirect activity measurements for *E. coli* in nonilluminated (A) and illuminated (B) systems. ▲, Total number of cells; △, metabolically active cells; ●, CFU on TSY; ○, CFU on EMB; ■, total uptake of [^{14}C]glucose; ◇, respired fraction of glucose taken up; □, assimilated fraction of glucose taken up.

ever, the illuminated systems showed a sharp decrease in the total uptake of glucose during the experiment (Fig. 2).

When we differentiated the fractions of assimilated and respired [^{14}C]glucose during the survival experiments, we observed that in the illuminated systems, the decrease in glucose assimilation occurred more rapidly than did $^{14}CO_2$ production (glucose mineralization) (Fig. 2). We also observed that the decrease in the total uptake of [^{14}C]glucose was produced parallel to the decrease in the respired fraction (Fig. 2). However, in the nonilluminated systems, total uptake and assimilated and respired fractions evolved in a parallel direction all through the survival experiments, and there was a slight increase during the period studied (Fig. 2).

We compared the assimilation and respiration percentages with respect to the total uptake and observed a great difference between the behavior of illuminated and nonilluminated cells in the survival studies (Table 1). In the nonilluminated systems, the assimilation and respiration percentages remained approximately constant throughout the experiments. While the percentage of assimilated glucose went from 79.3% at 0 h to 72.7% at 72 h, the percentage of respired [^{14}C]glucose went from 20.7% at 0 h to 27.3% at 72 h. On the other hand, in the illuminated systems the percentage of assimilated [^{14}C]glucose progressively decreased throughout the survival period studied. At the start of the experiment it was 79.7%, but after 72 h of incubation it was practically 0; therefore, the percentage of respired [^{14}C]glucose in illuminated systems rose from an initial 20.3% to practically 100% by the end of the survival experiment.

DISCUSSION

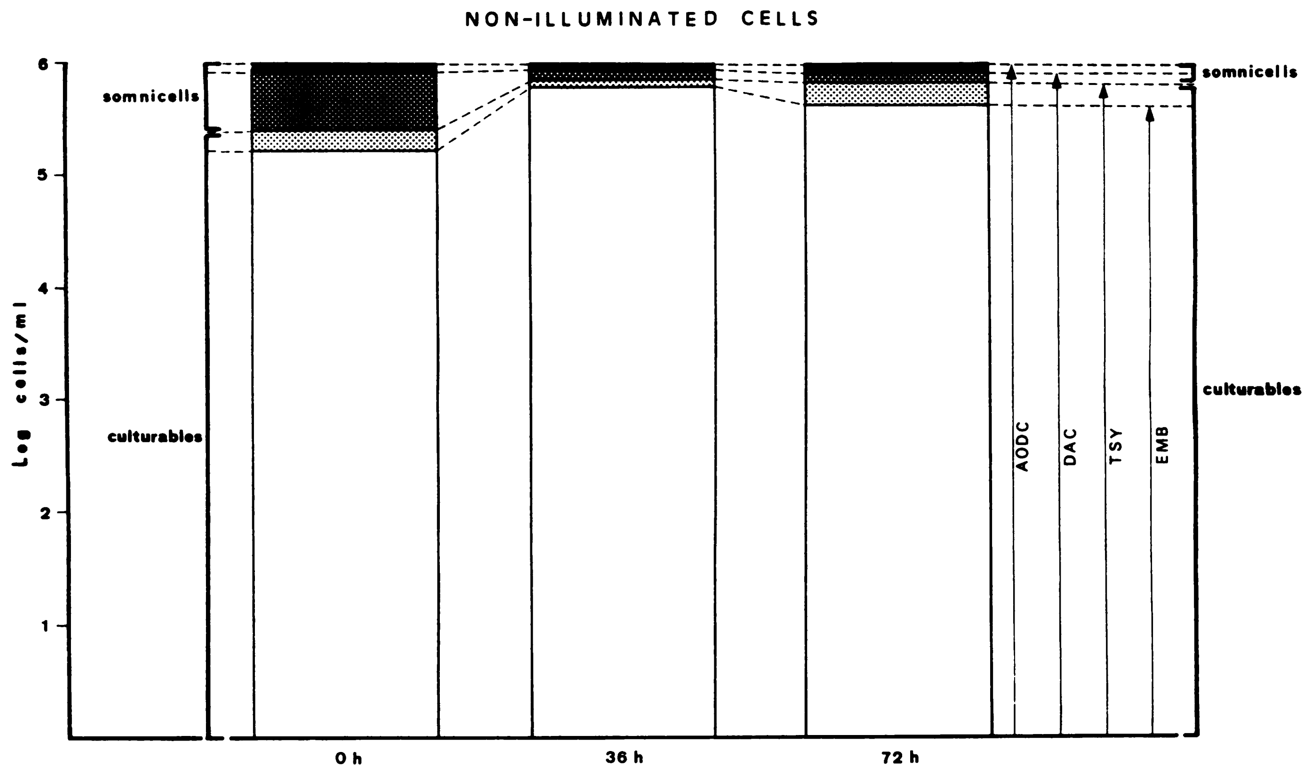
By comparing the evolution of *E. coli* cells in illuminated and nonilluminated systems (Fig. 2), we can deduce, as have other authors (4, 8, 9, 12, 16, 17, 19), that visible light has a negative effect on *E. coli* cells in fresh water. This is revealed by the decreases in the numbers of *E. coli* CFU and metabolically active cells (as determined by DAC method) in the illuminated systems.

On the other hand, if we take into account that the number of *E. coli* cells determined by AODC during our survival experiments remained constant in both illuminated and nonilluminated systems, we can affirm that visible light does not give rise to cellular lysis, at least not during the time studied. In accordance with the above, one of the effects of visible radiation on *E. coli* cells in fresh water is the progressive loss of their capacity to multiply in standard bacteriological media; however, they remain morphologically intact in the natural aquatic medium.

When we considered the number of metabolically active cells determined by the DAC method, we observed that in the illuminated systems, this number decreased during our experiments; however, this decrease was less than that of the number of CFU.

Therefore, since all counts remained approximately constant in the nonilluminated systems, and taking into account the evolutions of the different counts in the illuminated systems, we can conclude that visible light produces a progressive dormancy in *E. coli* cells in fresh water. This fact is clearly seen in Fig. 2.

Thus, at the end of the survival experiments in illuminated systems, four groups of *E. coli* cells exposed to visible light could be clearly distinguished on the basis of the four count methods employed (plate count on selective [EMB] and recuperation [TSY] culture media, DAC, and AODC). Group 1 is formed by those cells capable of forming colonies in the selective culture medium (EMB). These are cells which have not yet been affected by visible light and are



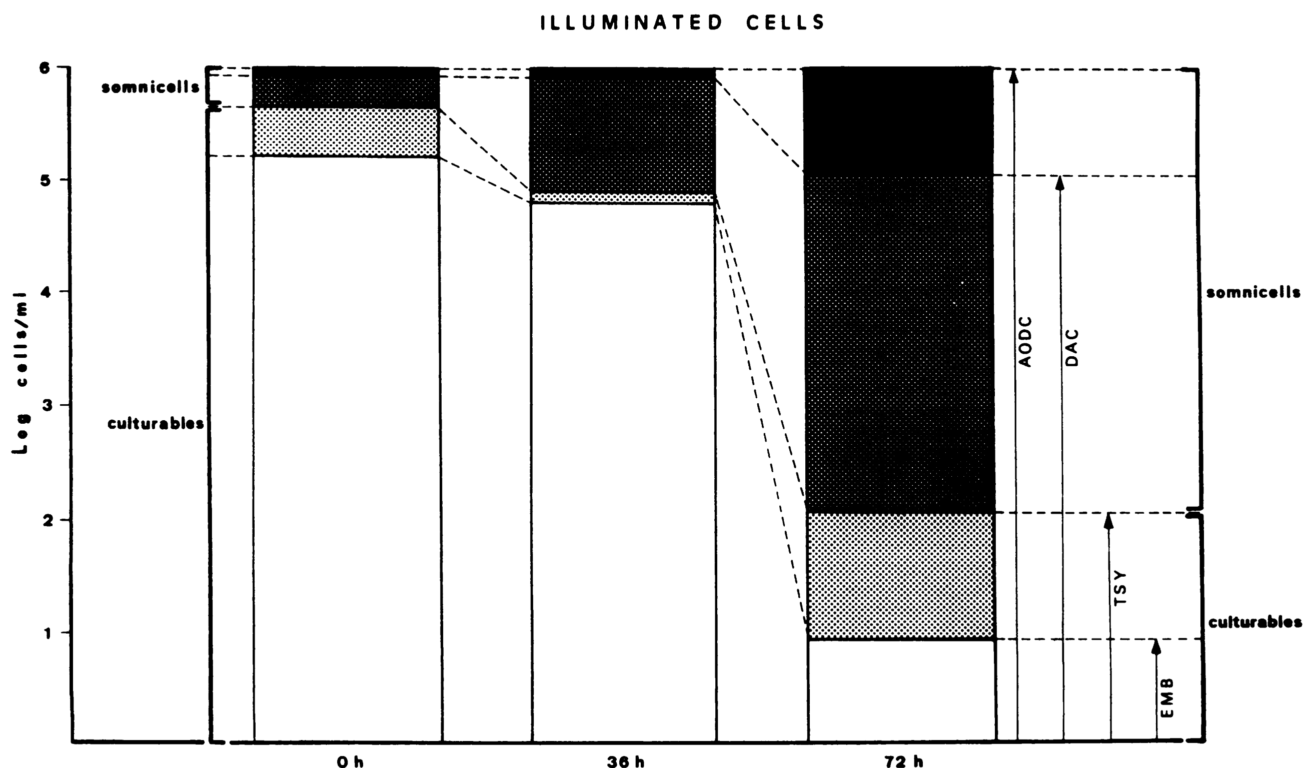


FIG. 3. Characterization of *E. coli* cells in survival stages of progressive dormancy in illuminated and nonilluminated systems at 0, 36, and 72 h of incubation.

TABLE 1. Percentages of assimilation and respiration of total glucose taken up in nonilluminated and illuminated systems

Time (h)	% assimilation in:		% respiration in:	
	Nonilluminated systems	Illuminated systems	Nonilluminated systems	Illuminated systems
0	79.3	79.7	20.7	20.3
12	58.7	52.3	41.3	47.7
24	61.2	29.4	38.8	70.6
36	62.0	10.1	38.0	89.9
48	65.9	9.6	34.1	90.4
72	72.7	0.0	27.3	99.9

culturable on any culture medium appropriate for *E. coli*. Group 2 contains cells which have been affected by light and so are not able to form colonies on a selective culture medium (EMB) but are capable of multiplying on a recuperative culture medium (TSY). These are injured *E. coli* cells whose recuperation is possible on TSY. These first two groups contain the culturable cells. Group 3 contains *E. coli* cells exposed to visible light and which are incapable of forming colonies in culture media but which still have a certain metabolic activity detectable by the DAC method. Group 4 contains *E. coli* cells affected by visible light to such a point that they are not culturable and do not undergo any type of metabolic activity detectable by the DAC method but which remain morphologically intact and can therefore be enumerated by the AODC method. Groups 3 and 4 contain the somnicells, which are not culturable cells. Figure 3 shows the characterization of *E. coli* cells in survival stages of progressive dormancy in illuminated and nonilluminated systems.

In view of our results, different survival strategies in illuminated and nonilluminated cells can be clearly observed. These differences are also shown in the results obtained by examining the uptake of [^{14}C]glucose by these *E. coli* cells in illuminated and nonilluminated systems (Fig. 2). While the uptake of [^{14}C]glucose underwent a slight increase in the nonilluminated systems, a progressive decrease in the uptake capacity of glucose was observed in the illuminated systems; therefore, we can deduce that in the illuminated systems of our survival experiments there was a progressive inhibition of active transport of glucose to the interior of *E. coli* cells as a result of their exposure to visible light.

When we took into account the differentiation between the assimilated and respired fractions of the total [^{14}C]glucose incorporation (Fig. 2), we also observed a different behavior between the illuminated and nonilluminated systems. From these results, we deduce that the effect of visible light is stronger on biosynthetic (or assimilative) processes than on degradative ones ($^{14}CO_2$ production), since during the survival experiments in illuminated systems, the decrease in the assimilated fraction took place more rapidly than in the respired fraction, which decreased parallel to the total uptake of glucose (Fig. 2). Moreover, the percentage of assimilation with respect to the total [^{14}C]glucose taken up decreased progressively in the illuminated systems of survival experiments, whereas the respiration percentage ($^{14}CO_2$ production) increased proportionally (Table 1). Therefore, we can deduce that light, apart from the aforementioned negative effect of glucose transport to the interior of *E. coli* cells, gives rise to the inhibition of certain assimilative (or biosynthetic) processes in *E. coli* cells.

When we compared the results obtained for the decrease in the number of CFU (on TSY and EMB) with the decrease in total uptake of [^{14}C]glucose during all the survival experiments in illuminated subsamples (Fig. 2), we observed a certain parallel between both evolutions. This leads us to think that the inability of *E. coli* cells to take up substances in fresh water due to the action of visible light could be a limiting process of the ability of these cells to multiply in standard bacteriological culture media. In this respect, it is important to underline the effect that the aforementioned inhibition of biosynthetic (or assimilative) processes can have on the capacity to form colonies in illuminated systems. Another important fact is that the greatest decrease in the number of metabolically active cells occurs at 72 h, when the total uptake of [^{14}C]glucose reached undetectable levels; therefore, we can deduce that the inability of *E. coli* to take up substrates is a decisive factor in the metabolic inactivation of these cells in natural fresh water exposed to visible light.

In view of these results, it would be extremely interesting to study in depth the physiological variations that accompany the survival stages of progressive dormancy as a result of the action of light on the survival of *E. coli* and of other enteric bacteria commonly used as bacterial indicators of fecal pollution in natural waters.

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