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Laser-induced thermal stress and the heat shock response in neural cells

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Background   The Ho: YAG laser is used extensively in orthopedic surgery. It offers a minimally invasive method of ablating tissue with precision. Previous studies have explored the effects of laser use on temperature during experimental foraminoplasty. To date, there has been limited work on the effects of thermal stress on cells in this context.

Material and methods   Cells were exposed either to heated medium or the Ho: YAG laser in the high-power mode. Heated medium was used as a stressor by (I) exposing groups of cells to a constant temperature of 45°C for varying lengths of time: 5, 10, 15 and 20 min, and (II) exposing cells for a fixed length of time (5 min) to varying temperatures: 45°C, 55°C, 65°C with a control treated at 37°C. A third group was subjected to direct laser treatment. The effects of the treatments were assessed using trypan blue staining as a measure of viability and immunocytochemistry was used to measure changes in heat shock protein (HSP) expression.

Results   There was a negative correlation between cell viability and HSP expression, and between cell viability and the severity of the treatment.

Interpretation   Our findings suggest a possible role for the Ho: YAG laser in spinal foraminoplasty based on the high level of cell viability in the treatment regimen that most closely mirrored the clinical application of the laser.

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As the use of lasers in surgery proliferates, it is essential that their application is both safe and effective. The Holmium: Yttrium Aluminium Garnet (Ho: YAG) laser has been used extensively in orthopedic surgery, a major potential application being for endoscopic spinal foraminoplasty. It offers the ability to ablate tissue with precision and less morbidity as compared to conventional techniques, which may involve extensive spinal exploration.

Previous studies have explored the effects of laser use on temperature during experimental foraminoplasty (Hafez et al. 2001). While the efficacy of the laser in the ablation of osseous and chondral tissue is not in question, concerns exist about the effect of heat generated on neighbouring neural tissue. As the Ho: YAG laser is a source of thermal energy, it may cause thermal stress and cell death, both of which are known to be induced by heat (Santoro 2000). To be able to study these processes, it was necessary to simulate the cellular environment and to examine the level of stress encountered.

The use of in vitro models for the exploration of physiological processes is well established, with various models of human and animal cells having been described. The main cellular components of neural tissue are Schwann cells, which are highly differentiated cells involved in the formation of myelin. The degree of differentiation and abundance of connective tissue make them difficult to isolate from adult mammalian peripheral nerves (Ansselin et al.1998).

Cells produce heat shock proteins (HSP) in response to stress caused by heat, poisons or signals from nerves or hormones. These heat shock proteins are sometimes called molecular chaperones, because their role is to protect the cell and

usher other proteins around, within the cells (Henderick and Hartl 1993). The process by which cells respond to stress is called the heat shock response. It was first observed in fruit flies and later in yeast (Santoro 2000). Even under non-stressed conditions, low baseline levels of chaperones are essential for prevention of newly made proteins from clumping together or being degraded before they can assume their final three-dimensional structure (Morimoto 1993). Exposure of cells to stress, whether it be heat stress or oxygen deprivation, as occurs in infection, results in the production of chaperones with the purpose of ensuring the maintenance of vital proteins. HSPs are highly conserved and have been classified into families according to molecular size. One of the most studied HSP families is the 70-kDa HSP, HSP 70.

We studied the effect of thermal stress (as would be encountered in laser surgery) on a mixed population of Schwann cells and fibroblasts in culture. This work was prompted by the need to understand the effects of the Ho: YAG laser and heat at the cellular level, using novel means such as HSP expression. We hypothesized that thermal stress would precipitate the production of HSP 70 comparable to that induced by the Ho: YAG laser. Differences in length of exposure and intensity of the stressful stimulus were studied.

Material and methods

We used 6 Wistar rats, each aged 6 weeks. Following killing, the whole animal was disinfected, and thereafter both sciatic nerves of all 6 animals were dissected out using aseptic technique.

Preparation of the neural tissue for culture

Using a surgical loop (Carl Zeiss, Germany), the epineurium was stripped off each nerve. The nerves were then divided into small pieces (1 mm^3) using a scalpel blade. The nerve tissue explants were left in a sterile Petri dish containing DMEM and incubated at 37°C for 3 days. 18 h before dissociation, 0.1% collagenase/dispase (Sigma Aldrich, Poole, Dorset, UK) was added to the explants to aid the digestion of collagenous tissue.

On the fourth day, dissociation of the explants was completed by repeated gentle trituration using a pipette of 1 mm diameter. The explants were then centrifuged, resulting in a single pellet composed of Schwann cells from all the initial animals. This pellet was then resuspended and used to seed a large cell culture flask (75 cm^2). The flask was incubated at 37°C in 95% air / 5% CO_2. The medium was changed every 3 days after a monolayer had been established. At confluence, the cells were passaged by being washed in phosphate buffered saline (PBS), trypsinized and reseeded into two flasks. Once the third passage (P3) had been reached, the experimental protocol was carried out, thus ensuring that all cells used in the experimental phase of this study were at P3.

Cell culture and maintenance

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. Routine cell culture was carried out under aseptic conditions in a class II microbiological safety cabinet (Crowthorne Hi-Tec Services Ltd., UK). Vented plastic cells, culture flasks and multiwell plates (Triple Red Ltd., UK) and sterile, single-use plugged disposable serological pipettes and plastic universal tubes (Bibby Sterilin, Staffs., UK) were used.

Cell culture reagents and media were obtained from Life Technologies (Poole, UK). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin G, 50 μg/mL streptomycin and 0.3 μg/mL amphoterecin B. Trypsinization was carried out using Trypsin-EDTA (1×) in HBSS without calcium and magnesium (Life Technologies).

Experimental design

Cell cultures were divided into three experimental groups according to treatment: Group I was subjected to a constant temperature (45°C) for varying lengths of exposure (5, 10, 15, and 20 min). Group II was subjected to a constant length of exposure (15 min) but at varying temperatures (45, 55 and 65°C). In the case of experimental groups I and II, a single control group was used. This was maintained under normal culture conditions: 37°C. Group III was subjected to direct exposure to the laser, in high-power mode: 2.1 mm at a setting of 0.01 kJ, 1.0 joules/pulse and 10 pulses/sec.

Exposure of cells to heat stress via heated medium

Experimental group I. At confluence, the cells were trypsinized and then counted using an improved Neubauer hemocytometer. The cells were then plated at a density of 9×10^4 cells/well onto 6sets of 6-well plates. Cells were then left overnight to adhere to the surface of the culture plates.

Heat stress was delivered by a change of culture medium, with the new medium being at the desired experimental temperature of 45°C. The medium was heated in a water bath, with the temperature being modulated by a thermocouple. The cells were maintained at 45°C for periods of 5, 10, 15 and 20 min following the change of the medium, by use of a second incubator preset to 45°C. The wells were then washed with sterile PBS. On the first two plates, the cells in 3 out of 6 wells were trypsinised and cell viability was estimated using the trypan blue exclusion method. The other 3 wells were washed with PBS and attached cells were directly stained with trypan blue for 2 min and then washed with PBS.

Using an inverted light microscope (Olympus), we examined cells in the wells morphologically and estimated viability using the trypan blue exclusion method. The untreated control (a 6-well plate kept at 37°C) was assessed in the same manner as above. Of the remaining 4 sets of 6-well plates, 2 were used to investigate HSP 70 expression and the other two were used to phenotype the Schwann cells by means of a polyclonal antibody against S100 Sigma (Poole, UK).

Experimental group II. Using the same seeding protocol as above, another six 6-well plates were incubated until confluence. On each culture plate, 3 of 6 wells were trypsinized and the cell viability was estimated by the same methods outlined for experimental group I.

Using an inverted light microscope (Olympus), we examined cells in the wells morphologically. The number of viable cells in a set area was also counted. The control, a 6-well plate kept at 37°C, was treated in the same way as described above. The other plates were incubated for 15 min at temperatures of 45°C, 55°C or 65°C, with a separate incubator being used to maintain cells at the desired experimental temperature.

Assessment of cells following thermal treatment

Viability testing of cells. We counted viable cells using the trypan blue exclusion method. Trypan blue is a polar dye that is not able to cross intact cell membranes but crosses the membranes of necrotic cells and apoptotic cells undergoing secondary necrosis. It is therefore only partially informative in elucidating the mechanism of cell death but it is a useful, rapid and simple screening assay for viable cells. Following trypsinization and resuspension in a set volume of medium, the cell suspension was transferred to a clean Eppendorf tube. Equal volumes of the cell suspension and trypan blue were mixed and the suspension was allowed to stand for 1–2 min. 5 μL of the cell suspension was added to the hemocytometer chamber. The live cells present in 25 squares of the hemocytometer were counted. The samples were also examined for morphological changes.

HSP immunocytochemistry. Following long immersion in xylene and alcohol, the sections to be stained for HSP 70 were immersed in 2% hydrogen peroxide in methanol for 10 min after which they were washed in running tap water. The sections were ringed with a PAP (peroxidase antiperoxidase) pen, placed in a humidity chamber and covered with tris-buffered saline (TBS). Normal horse serum was applied for 10 min and then removed by draining. This was followed by the application of mouse monoclonal antibody, anti HSP 70 (Ab Cam, Cambridge, UK) at 1:3000 for 60 min at room temperature. After washing with triamino buffered saline (TBS), biotinylated horse anti mouse antibody was applied and left for 40 min at room temperature before being rinsed off.

AB complex (Vector Laboratories, Peterborough, UK) was applied at 1:100 at room temperature and then rinsed off. This was followed by the application of diaminobenzidine (DAB) using SK4100 kit (Vector Laboratories, Peterborough, UK) at room temperature. A wash in TBS then followed. Counterstaining with hematoxylin was carried out. The antigenic sites stained dark brown and the nuclei stained blue.

Phenotyping of Schwann cells. We performed Schwann cell phenotyping using fluorescence immunocytochemistry with anti-S100, which is
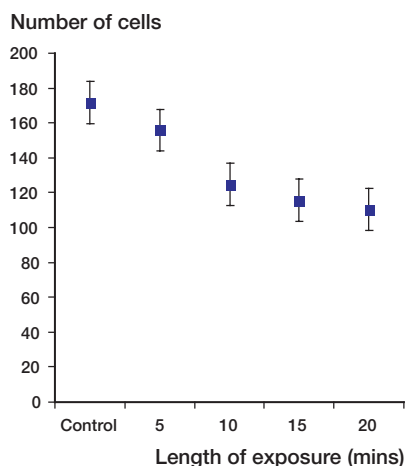
Figure 1. Viability of schwann cells following exposure to 45°C for varying lengths of time.
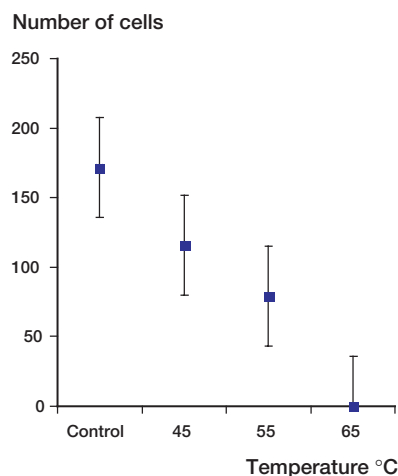

Figure 2. Viability of schwann cells following exposure for 15 minutes to different temperatures.

a polyclonal anti S100 rabbit antibody against a specific cytoplasmic protein found mainly, but not exclusively, in human cells of neural origin. Schwann cells were seeded on slide chambers for 48 h at a density of 9×10^4 per chamber, after which washing was performed three times by immersion of the slide in MEM-HEPES buffer. Fixation and permeabilization of the cells was done using 4% paraformaldehyde for 10 min. This was followed by incubation with 25 μL primary antibody (anti-S100 at 1:100) (Sigma, Poole, UK) for 1 h at room temperature. Secondary antibody conjugated to fluorescein isothyocyanate (FITC) (Sigma) at a dilution of 1:100 was put on each slide and incubated for 1 h at room temperature. Following three final washes, the slides were mounted in Citiflor with a coverslip and sealed with nail varnish. They were examined using a fluorescent microscope with an excitation wavelength of 494 nm and an emission wavelength of 517 nm.

Experimental group III. We prepared microscopy slide chambers in the same way as the culture well plates, with cells being seeded at the same density. The cells were exposed to a Ho: YAG laser (2.1 mm), at a setting of 0.01 kJ (1.0 joule/pulse and 10 pulses/sec). The cells were then washed with PBS to remove culture medium. Both treated and untreated control slides were stained with 4% trypan blue and assessed visually. Quantitative studies were not done in this group.

Statistics

The effect of each treatment regimen was analyzed by performing a one-way analysis of variance (ANOVA). This was carried out on groups I and II only, as the results obtained from direct lasing in experimental group III were of a qualitative nature.

Results

The results obtained were both qualitative and quantitative. Quantitative results were in the form of the number of viable cells after set treatments (Figures 1 and 2). Qualitative results were in the form of observations as to whether HSP 70 and S100 proteins were expressed or not. A negative correlation was observed between the number of viable cells and the length of time cells were exposed to the heated medium (Figure 1). Similarly, when temperature was increased and the length of time was kept constant, a negative correlation between cell viability and temperature was observed (Figure 2). In both cases, a one-way analysis of variance was used in assessing the results, with $p < 0.001$ in both cases. The control reflected the number of viable cells that were present in a set area under conditions of 37°C with no treatment regimen.

An increase in the length of time for which cells were exposed to a stressful stimulus was subjec-
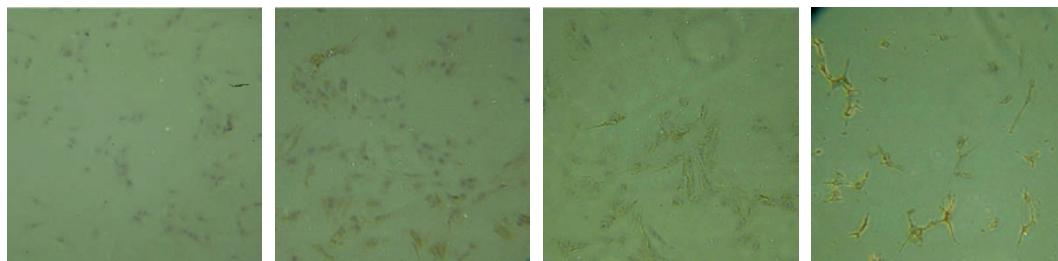
Figure 3. HSP results following exposure to 45°C for 5, 10, 15 and 20 minutes.
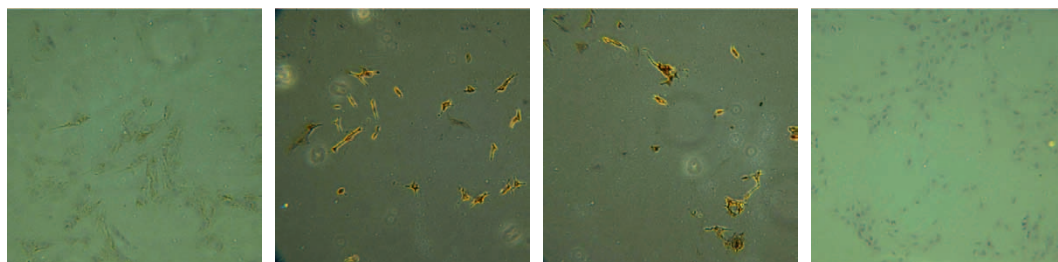

Figure 4. HSP expression following exposure for 15 minutes to temperatures of 45, 55 and 65°C with a control at 37°C.
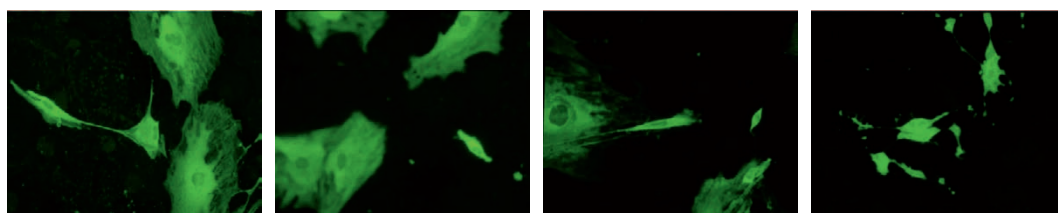

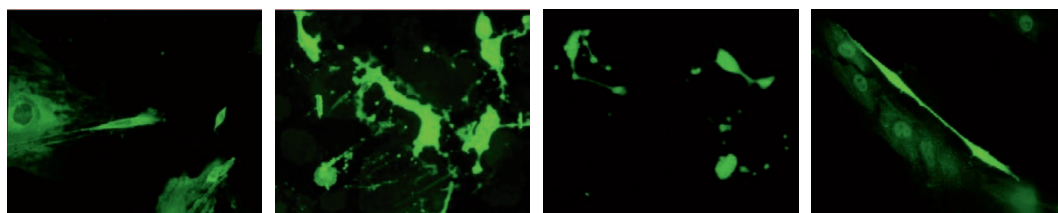Figure 5. S100 results following exposure to 45oC for 5, 10, 15 and 20 minutes.

Figure 6. S100 expression following exposure for 15 minutes to temperatures of 45, 55 and 65°C, with a control at 37°C.

tively noted to cause an increase in HSP expression, though this tailed off by 20 min (Figure 3d). This may be because exposure beyond a certain threshold point is lethal to cells. Dead cells are not capable of expressing HSP.

We found a decrease in HSP expression at the extremes of temperature (Figure 4). This decrease was not in the expression of HSP per se but in the number of viable cells available to express HSP, reflecting the lethality of the temperature. Cells

were stained for S100. The staining confirmed the retention of phenotypic characteristics, despite the thermal stress. A subjective assessment of the morphology showed that with increase in temperature and length of exposure, there was ruffling of cell membranes. This is illustrated best in Figures 5d and 6b.

When staining for HSP 70 immediately after laser treatment (Figure 7a) we found no expression of HSP. When the cells were reincubated for 3 h
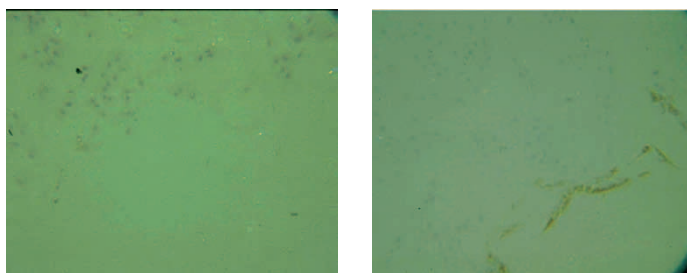
Figure 7. Minimal HSP expression following direct lasing, with immediate fixation (left) and significant HSP expression following direct lasing, with fixation 3hours post lasing (right).

following laser treatment and then stained, HSP 70 expression was present (Figure 7). This suggests that an interval is required to allow synthesis of HSP.

Discussion

The overriding aim of this study was to develop a cellular model to investigate the effects of thermal stress which may be encountered by tissues in the surgical use of the Ho: YAG laser. During laser foraminoplasty, the dorsal root ganglia and nerve roots may be at risk of being damaged, as tissues in close proximity reach temperatures that are higher than physiological temperature when lasers are used (Konno et al. 1994).

We found that there was a negative correlation between thermal stress and the number of viable cells in culture plates. We observed both qualitatively and quantitatively that changes occurred as both the level of thermal stress and the period for which the cells were exposed increased. Cells on microscope slides that were subjected to laser treatment mirrored these changes. Although it was not possible to quantify the effects on the laser-treated cells, we found a qualitative difference.

As previous work has shown, a reduction in temperature occurs at the point of lasing with the use of saline irrigation (Hafez et al. 2001). The complexity of laser attenuation means that it cannot accurately be modelled for every situation. Nonetheless, a relative measure of attenuation can be obtained by reference to previous work (Blomey et al. 1995). The degree to which the effect of the laser is attenuated is dependent on variables, which include mode (contact or non-contact) and

the viscosity of the medium. The important principle underlying the safe use of the Ho: YAG laser is the interposition of a liquid medium between or around the laser and the area being treated, although this attenuates the effective penetration depth of the laser.

Saline irrigation during laser treatment keeps the temperature of the surrounding tissues at 37°C (Hafez et al. 2001). Exposing the cultured cells to 45°C for 5 min, which is fully 8°C above the clinical situation, resulted in a high level of cellular viability after treatment when compared to the controls. Two conclusions can be drawn from this. Firstly, during surgery it is unlikely that the same point will be lased continuously for more than five minutes and secondly, the tissue temperature induced by the laser would be much lower than this when augmented with irrigation. The real situation is thus less stressful than the doses applied in this series of experiments (Hashieh et al. 1997).

It was our intention to design the experimental regimens to be harsher than would be expected in vivo, as this would account for any extremes of treatment that might occur. The untreated controls were kept at 37°C, which is the temperature of the surrounding tissue during experimental irrigated laser assisted foraminoplasty (Hafez et al. 2001). Samples were stained immunohistochemically for S100 proteins to confirm the Schwann cell phenotype. Although the presence of S100 protein is not tissue-specific, it is recognized as a marker of neural cells. Recent work (Okada et al. 2003) has shown S100 to be a molecular chaperone also, and a member of the Hsp70/90 family. So, although only a subjective observation, it was noted that the control sample expressed less fluorescence than thermally treated samples, which is in line with

what would be expected if S100 was indeed also a measure of cellular stress.

We measured cell viability by counting trypsinized cell samples previously stained with trypan blue. This method does have its limitations, which include the fact that only a small proportion of cells are sampled and that the counting is done manually. Further validity may be given to the results by the use of a more accurate method of assessing cell viability, such as the 3H-thymidine incorporation assay, which avoids the use of trypsin and its stressful effects prior to quantification. Nevertheless, it should be recognized that any stressful effect induced trypsin was uniform throughout all the samples.

From a wider standpoint, nerves were found to discharge in response to a 'burst' of the Ho: YAG laser (Qadir and Kennedy 1993). This nerve activity became stronger in proportion to the proximity of the nerve. The significance of these previous findings for our work is twofold. Firstly, the fact that our work used neural cells and not whole nerves immediately highlights a difference in approach. Whether a process occurring at an ultrastructural level can be repeated at the cellular level is not immediately apparent. Due to the important role that the anatomical structure of a nerve plays in the physiological function of the nerve, a cell culture system may be unable to accurately reflect the relationship between nerve discharges and laser impulses. The triggering of nerve impulses in response to the use of a laser (Qadir and Kennedy 1993) has not been shown to be deleterious to the nerve; it may even be used as a 'natural' safety mechanism. This is because as nerves are naturally designed to discharge, failure to do so during the use of a laser may suggest injury. Secondly, we believe that shock waves have a role in the mechanism of action of the Ho: YAG laser, even though this contradicts previous work (Qadir and Kennedy 1993). When cells seeded on culture plates were subjected to the laser and then processed for HSP 70 expression, we observed a circular area having no cells. This central area was bounded by a 'ring' of cells expressing HSP 70. A thermal mechanism alone cannot explain this because the procedure was carried out in culture medium, so the surrounding fluid would have caused an attenuation of the laser (Blomey et al.

1995). This has led us to conclude that the area in which cells were seen to be detached was due to a shockwave effect.

As the effects of direct laser treatment are attributable to both a thermal and a shock wave mechanism, quantitative measurements were not made, as this work was concerned mainly with the quantifiable effects of thermal stress (Sonden et al. 2000). The presence of a shock wave effect would therefore act as a statistical confounder for any quantitative extrapolations. However, we think that the effects of the shock wave would best be modeled using a three-dimensional osseous matrix, which—although it is beyond the immediate remit of the study—would be a consideration for future work.

The temperature has been found to be 150°C during direct laser treatment without continuous irrigation (Qadir and Kennedy 1993). In this situation, irrigation was used after the lasing and not during the procedure. Continuous irrigation results in much reduced peak temperatures (Hafez et al. 2001). The methods of irrigation obviously differ between the two previously named studies. The degeneration of nerve tissue observed by Qadir and Kennedy (1993) which was ascribed to temperature is, we believe, also due to the shock wave effect. The cells which were most affected and thus most likely to express HSP 70 were also subject to the most mechanical stress, caused by the shock wave effect. We postulate that these cells were both positive for HSP 70 and lost in the processing. We observed senescent cells with ruffled membranes and a flattened appearance. These would be classified as 'dead' using trypan blue staining. They may represent cells that have gone into a protective state, induced by the extremes in temperature (Santoro 2000). The significance of this observation lies in the fact that that it was made on cells that had undergone treatment at 65°C (Van Breugel and Bar 1993).

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