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SCK Tumor Cell Killing by Hyperthermia in the Presence of Heat Protector and Heat Sensitizer

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The present investigation aims at elucidating a possible mechanism of heat inactivation of SCK cells by comparing the kinetics of cell lethality and protein degradation in the presence of heat protector or heat sensitizer. The effect of heat sensitizer and protector was exhibited in both cell survival and protein degradation kinetics, the magnitude of the effect being much profound for the protector compared to the sensitizer.

A conclusion to be drawn from the present experiment is that there is no direct correlation between cell lethality and protein degradation. Rather, protein degradation, which might occur in the membrane, causes cell inactivation indirectly, possibly by altering the cellular environment. Accordingly, further studies are needed to get insight into the mechanism of cell inactivation by hyperthermia.

KEY WORDS: Hyperthermia, Cell survival kinetics, Protein degradation kinetics

Despite a great deal of information is known of the cellular and molecular responses of cells to hyperthermia, the mechanism(s) and primary target(s) responsible for cell death are unknown. Nevertheless, hyperthermia causes alterations in many physiological functions in the cell, such as DNA polymerase β activity (Spiro *et al.*, 1982), insulin-receptor binding (Calderwood and Hahn, 1983), and protein synthesis (Oleinick, 1979).

Furthermore, Many studies support the hypothesis that the plasma membrane is a critical target for heat cell inactivation. Hyperthermia has been reported to induce structural changes in cell membrane (Bass *et al.*, 1978; Lin *et al.*, 1973; Cost *et al.*, 1979). Cell survival studies combining hyperthermia with membrane-active agents such as alcohols (Li *et al.*, 1977) and polyamines (Ben-Hur *et al.*, 1978; Gerner *et al.*, 1980) further indicate that damage to membranes may be a primary lesion.

Critical targets other than the plasma membrane have been suggested for heat inactivation of cells. For example, hyperthermia may induce nucleolar changes (Simard and Bernhard, 1967; Love *et al.*, 1970), induce chromosomal aberrations (Dewey *et al.*, 1978), and cause alterations in DNA synthesis (Dewey *et al.*, 1980; Henle and Leeper, 1979; Mondovi *et al.*, 1969). In addition, hyperthermia inhibits protein synthesis (Dewey *et al.*, 1980; Henle and Leeper, 1979; Mondovi *et al.*, 1969), presumably by blocking the initiation step (Oleinick, 1979), and causes protein denaturation (Rosenberg *et al.*, 1971). Heat also causes an increased association of nonhistone chromosmal proteins with DNA (Tomasovic *et al.*, 1978; Roti Roti and Winward, 1987). The kinetics of this increase has been correlated with the thermodynamics of heat-induced cell inactivation (Roti Roti *et al.*, 1979). Similarly, a heat shock at 43°C for 1 hr causes a rapid but reversible loss of a 45 kD protein, while other high-molecular-weight proteins are induced (Bouche *et al.*, 1979).

In the present investigation we have studied the effect of hyperthermia on the cell survival and protein degradation in the presence of heat protector or heat sensitizer, aiming at elucidating

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mechanism of cell inactivation by heat.

Materials and Methods

SCK Tumor Cell Culture

SCK tumor cells, mammary carcinoma origin of A/J mouse, were cultured as described previously (Kang *et al.*, 1980).

Maintenance of Acidic pH

pH of the culture media was adjusted and maintained as described by Hahn and Shiu (1983). Normal (7.4) and acidic (6.7) pHs were obtained by adjusting the amount of sodium bicarbonate in the media. The adjusted pH was maintained by continuous gassing of 5% CO_2-air. The pH levels were reproducible from experiment to experiment.

Glycerol Medium

1M glycerol medium in RPMI 1640 medium supplemented with 15% bovine serum was filtered just prior to use. The cell were equilibrated with this medium 20 minutes before hyperthermia.

Hyperthermic Treatment

Prior to hyperthermic treatment, SCK tumor cells appropriately seeded in 35 mm T-flasks were replaced with normal, acidic or glycerol-media. Culture dishes thus treated were tightly capped, sealed with parafilm and inserted in plastic shelves were immersed horizontally in a constant temperature-circulating waterbath for desired durations.

Cell Survival Studies

Following hyperthermia, the cells were trypsinized and viable cells were counted using trypan blue exclusion method. Appropriate number of cells were then seeded onto culture dishes and were incubated for 7-10 days. On termination of incubation, the cells were fixed and stained with crystal violet and the colonies were counted for plating efficiency.

SDS-PAGE

Cells were labelled with 10 μCi/ml of ^{35}S-methionine in methionine-free RPMI 1640 medium for 1-2 hr at 37°C. At the end of this period, the medium was removed and the cells were washed three times with cold phosphate buffered saline and harvested in lysis buffer which contained 2% SDS. Labelled samples were dissociated by heating in boiling water for 3-5 min. The amount of porotein was determined by the method of Lowry *et al.* (1951). Equal amount or equal radioactivity of protein was directly loaded onto 7.5-10% SDS-polyacrylamide slab gels by employing the method of Laemmli (1970).

Autoradiography and Fluorography

After the SDS-PAGE, the gels were either autoradiographed or fluorographed as routinely.

Rate of Protein Degradation

Cells were labelled with 1 μCi/ml of ^{35}S-methionine for 15 hr and then washed three times with plain RPMI 1640 medium. To the cells were added either normal, acidic, or glycerol-medium and hyperthermic treatment was given at varying temperatures and durations. Rate of prelabelled protein degradation was determined by measuring the ratio of radioactivity of TCA-soluble products to that of TCA-insoluble materials (S/I ratio).

Results

Cell Lethality by Hyperthermia

Survival curves were obatined for the cells exposed to hyperthermia at 41-45°C for either 1-5 hr or 10-60 min in normal, acidic or glycerol media and survival curves at 42 and 43°C are shown in Figs. 1 and 2. The survival curves revealed that glycerol served as a good heat-protector, whereas acidic pH served as a heat-sentitizer. The protective effect of glycerol was found to be more profound over the sensitizing effect of acidic pH at all temperature ranges, except 41°C. The significance of this difference is obvious if we compare D_0 values, as shown in Table 1.

Inactivation Energy of Cell Lethality

Inactivation energies were calculated from cell survival data at each medium conditions (Table 1). From cell survival curve at each temperature, Do values were obtained in min. Since the relation K

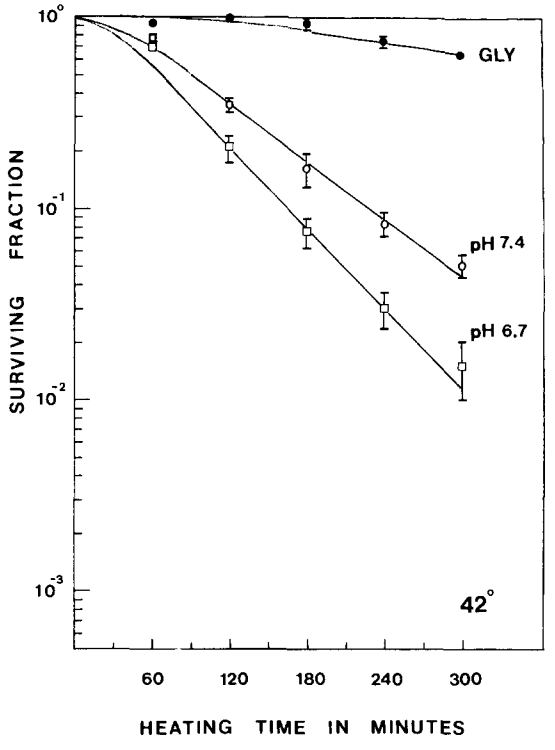

Fig. 1. Effect of heat-protector and heat-sensitizer on the SCK cell survival. The cells were exposed to 42°C for designated times under different medium conditions.

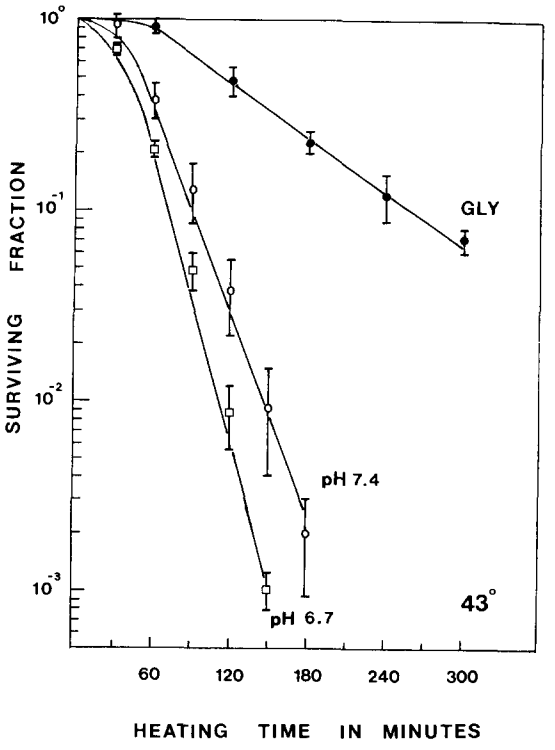

Fig. 2. Effect of heat-protector and heat-sensitizer on the SCK cell survival. The cells were exposed to 43°C for designated times under different medium conditions.

Table 1. D_0 and inactivation energy (ΔH) for SCK cells exposed to hyperthermia at 43 and 44°C under different medium conditions.

Heat	pH 7.4		pH 6.7		Glycerol	
	D_0 (min)	ΔH (kcal/mol)	D_0 (min)	ΔH (kcal/mol)	D_0 (min)	ΔH (kcal/mol)
43°C	21.3	239	12.0	190	91.7	317
44°C	6.4		5.6		17.6	

$= 1/D_0$ holds, inactivation energy H corresponds to 4.8 log $K_1/K_2/T_2/T_1$, which is derived from Arrhenius equation $K = Ae^{-H/RT}$. Comparison of the inactivation energies obtained at glycerol and acidic medium suggest that glycerol and acidic pH affect cell inactivation by different mechanisms. The break point in the Arrhenius plot appears different, being 42.5°C and 44°C for acidic pH and glycerol medium, respectively. Furthermore, inactivation energy itself shows quite a difference. For example, 239 Kcal/mol at normal pH decreases to 190 Kcal/mol at acidic pH and increases up to 317 Kcal/mol at glycerol medium, when the values are calculated at temperature

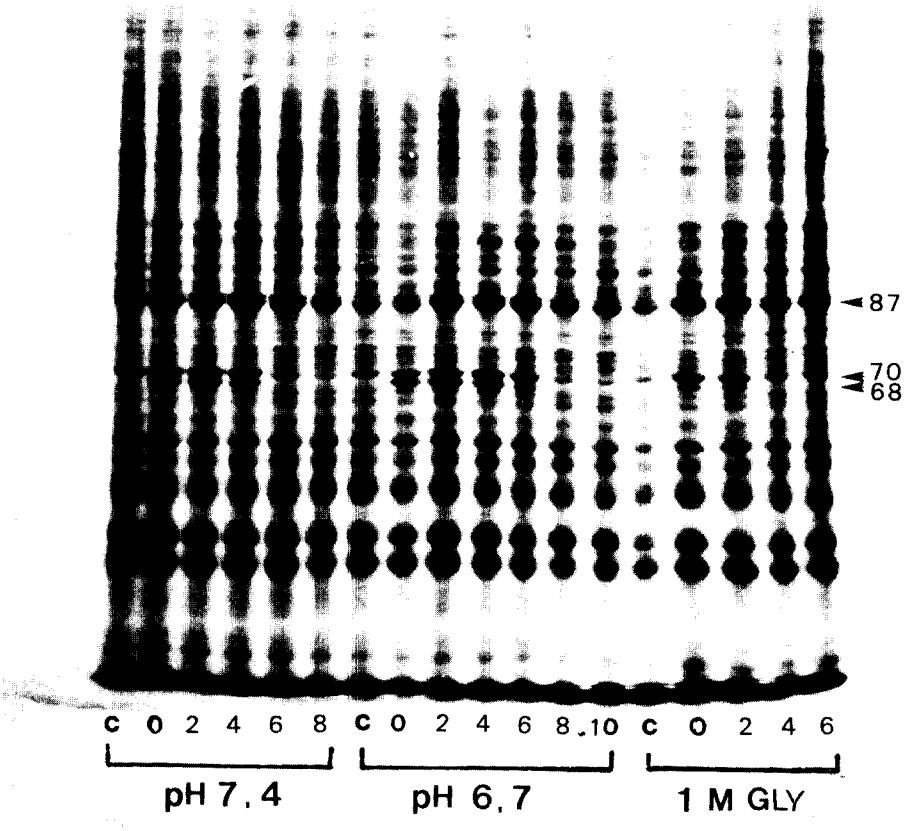

Fig. 3. Pattern of protein synthesis in the SCK cells exposed to 42°C for 1 hr under different medium conditions, followed by incubation for varying durations and labelling with ^{35}S-methionine for 2 hr.

range of 43-44°C.

Kinetics of $HSP_{68\ 70}$ and Heat Sensitivity

For the evaluation of change of heat sensitivity under different conditions, kinetics of protein synthesis, especially of $HSP_{68\text{-}70}$, were followed for up to 10 hr following hyperthermia at 41-45°C. Autoradiographic patterns for the rate of protein synthesis in the cells exposed to hyperthermia at 42°C and 43°C for 1 hr are shown in Figs. 3 and 4, respectively. Kinetics for the rate of $HSP_{68\ 70}$ synthesis, as expressed in terms of per cent of total proteins, at 42 and 43°C for 1 hr are shown in Figs. 5 and 6, respectively. Assuming that cell copes with the stresses by adjusting the time and rate of stress protein synthesis, it is very likely that the cells synthesize less stress proteins in the presence of heat protector than in the presence of heat sensitizer. Thus, the rate and time of stress protein synthesis are likely to be determined by the degree the cells comply with the encountered stresses.

Degradation of Protein by Hyperthermia

Since it is assumed that the cell inactivation might be brought about by some specific protein inactivation, cellular proteins were labelled with ^{35}S-methionine prior to exposing to hyperthermia under different conditions. The degree of protein degradation by hyperthermia was evaluated by the

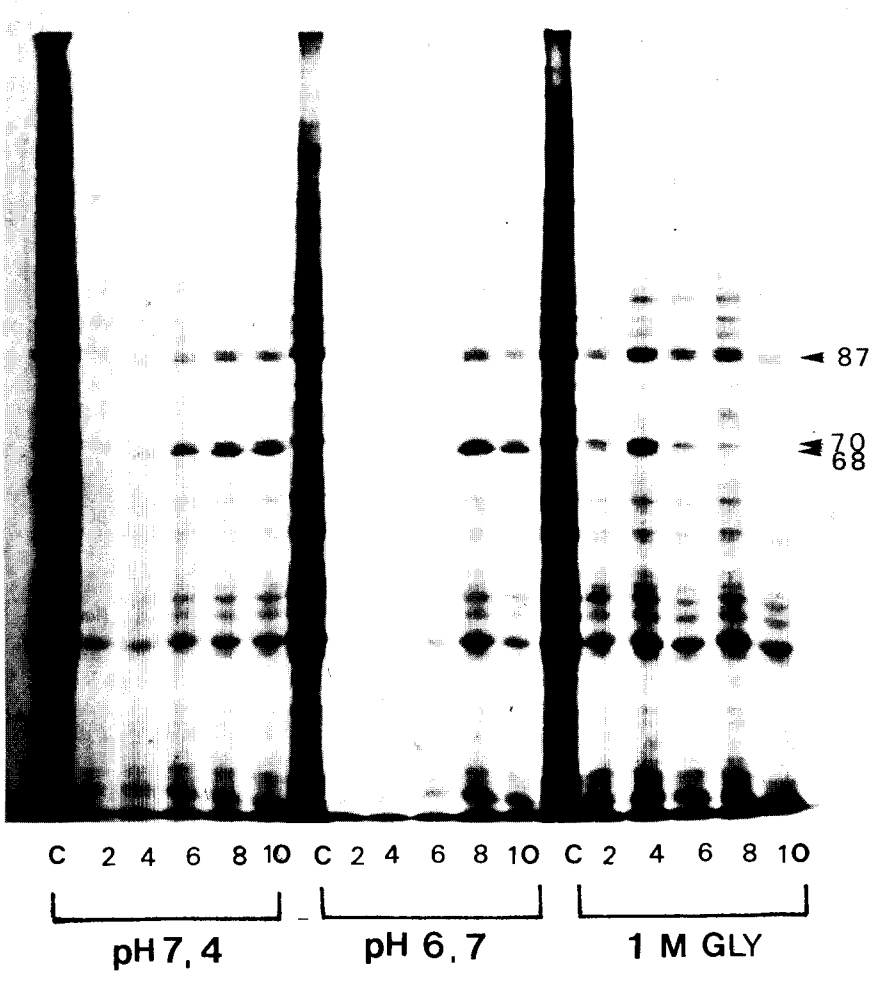

Fig. 4. Pattern of protein synthesis in the SCK cells exposed to 43°C for 1 hr under different medium conditions, followed by incubation for varying durations and labelling with ^{35}S-methionine for 2 hr.

ratio of TCA-soluble fraction over TCA-insoluble fraction (S/I ratio) at varying incubation durations. The S/I ratios obtained for hyperthermia at 43°C for 2 hr is shown in Fig. 7. As expected, rate of protein degradation decreased in the presence of heat protector, whereas the degradation increased in the presence of heat sensitizer.

Discussion

Since various studies have linked inactivation energies of cell inactivation with those of proteins, the present investigation aimed at elucidating whether correlation really exsts between kinetics of cell inactivation and that of protein degradation in the presence of heat protector and heat sensitizer.

Cell survival data revealed that the effect of both heat protector and sensitizer appeared in the whole range of hyperthermia examined, the magnitude of the effect being profound for protector than for sensitizer. D_0 values as well as inactivation energies for each occasion clearly explained

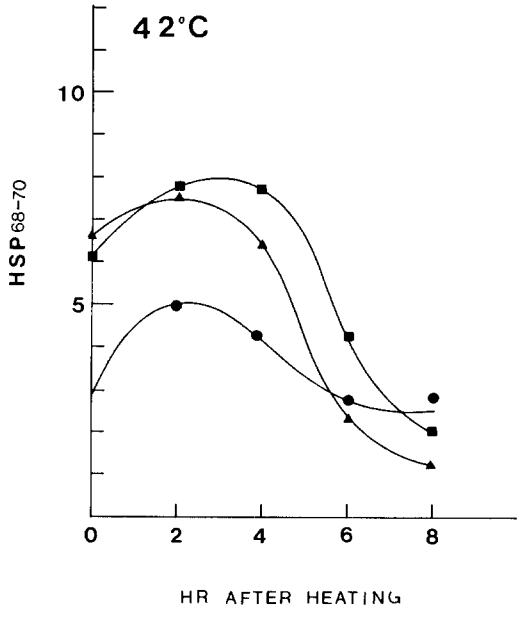

Fig. 5. Kinetics of $HSP_{68\ 70}$ synthetic rate, expressed as percent of total protein, in the SCK cells exposed to 42°C for 1 hr under different medium conditions. ▲—▲, pH 7.4; ■—■, pH 6.7; ●—●, glycerol medium.

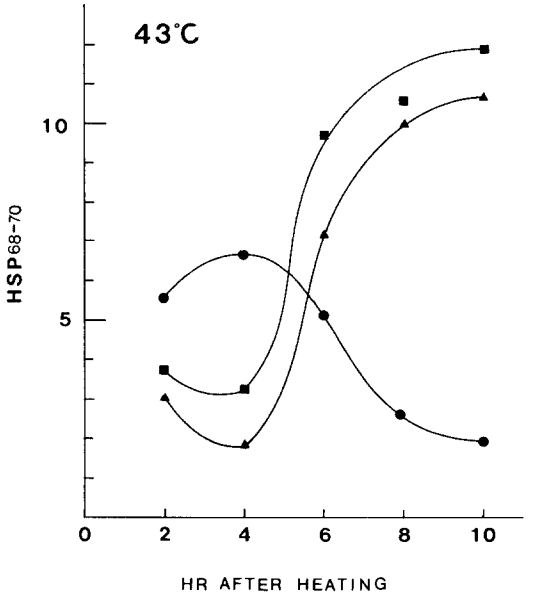

Fig. 6. Kinetics of $HSP_{68\ 70}$ synthetic rate, expressed as percent of total protein, in the SCK cells exposed to 43°C for 1 hr under different medium conditions. ▲—▲, pH 7.4; ■—■, pH 6.7; ●—●, glycerol medium.

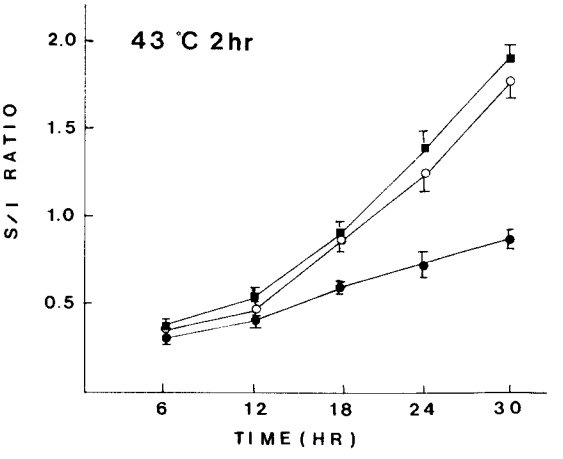

Fig. 7. Degradation rate of labelled proteins in the SCK cells exposed to 43°C for 2 hr under different medium conditions. Degradation rate is expressed in terms of S/I ratio (TCA-soluble fraction/TCA-insoluble fraction). ○—○, pH 7.4; ■—■, pH 6.7; ●—●, glycerol medium.

the situation. For instance, D_0 values at 43°C were 21.3, 12.0, and 91.7 min in normal, acidic and glycerol media, respectively. Similarly, cell inactivation energy in normal medium was calculated to be 239 Kcal/mol and it decreased to 190 Kcal/mol in acidic medium, whereas it increased to 317 Kcal/mol in glycerol protected medium.

Initially, it was predicted that the kinetics of cell survival following hyperthermia would correspond with either those of protein synthesis or those of protein degradation, at least for some of the stress proteins. However, the pattern of kinetics of protein synthesis was found not the same as those of cell survival following hyperthermia. Accordingly, the possibility that cell survival might be attributable to the decrease in the rate of protein synthesis was not likely and cell inacivation was not affected directly by the decrease in the protein synthesis.

Second possibility that cell inactivation might be induced by the increased protein degradation by hyperthermia was partially proved in the present experiment, the kinetics of the cell survival and that of at least HSP_{68-70} degradation being similar. Protein degradation by hyperthermia might be attributable to the affected membranes as wel as

intracellular membrane structures. Altered membrane by heat may induce changes in the cellular environment which causes changes in the enzyme activity that might lead to protein degradation.

Particularly, low pH was suggested to sensitize protein molecules by changing polar interactions or by ionizing active center of the protein molecules (von Ardenn, 1972). On the other hand, glycerol is thought to stabilize protein molecules by stabilizing hydrophobic interactions between nonpolar head groups in the molecules (Oleinick, 1979), and it adsorbs to the surface of the protein molecules occupied usually by the water molecules (Webb, 1963). Accordingly, important cellular structures such as membrane or microtubules could be stabilized by glycerol treatment (Fekete, 1978; Zabreacky and Cole, 1977). Therefore, it is likely that both heat-sensitization by low pH and heat-protection by glycerol occur at the level of protein-lipid on the membrane and that cell lethality might be produced by secondary changes such as ion concentration (Rosenberg *et al.*, 1971).

In conclusion, the present data indicate that cell lethality by hyperthermia does not correlate with protein degradation and that protein degradation indirectly affects cell lethality. Clearly, more data are needed before generaliztions can be made. However, the ability to modify cellular heat sensitivity by use of pH and glycerol can provide an important tool for the study of thermal biology.

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熱保護劑와 熱增感劑의 존재하에서 溫熱處理에 의한 SCK 腫瘍細胞의 致死機作

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본 연구는 온열처리에 의한 세포치사의 mechanism을 밝히기 위해서 heat sensitizer인 low pH와 heat protector인 glycerol을 이용하여 cell lethality와 단백질의 분해 kinetics를 검토한 것이다.

41-45°C의 온열처리 중에서 41°C를 제외한 전 온도범위에서 sensitizer와 protector의 효과가 뚜렷이 나타났으며, protector의 효과는 cell lethality와 단백질분해 모두에서 sensitizer의 효과에 비해서 현저히 나타나서 sensitizer와 protector의 작용기작은 서로 다를 것으로 생각되었다. 즉, 43-44°C에서 cell inactivation energy는 정상, low pH, glycerol 상태에서 각각 239, 190, 317 kcal/mole의 값을 보였다. 단백질분해 kinetics의 경우에도 대체적인 경향성은 cell inactivation kinetics와 유사하였으나, 직접적인 연관성은 발견할 수 없었다.

이와 같은 결과로 미루어 볼 때, cell lethality와 단백질 분해의 mechanism 사이에 직접적인 관계는 없고, 주로 막단백질로 추정되는 단백질의 inactivation에 의한 세포내 환경의 변화에 의해서 2차적으로 세포치사가 일어나는 것으로 추정할 수 있으며, 정확한 mechanism을 밝히기 위해서는 DNA polymerase를 비롯한 몇가지 가능한 표적에 대한 연구가 이루어져야 할 것으로 사료된다.