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Protein-Directed Modulation of High-LET Hyperthermic Radiosensitization

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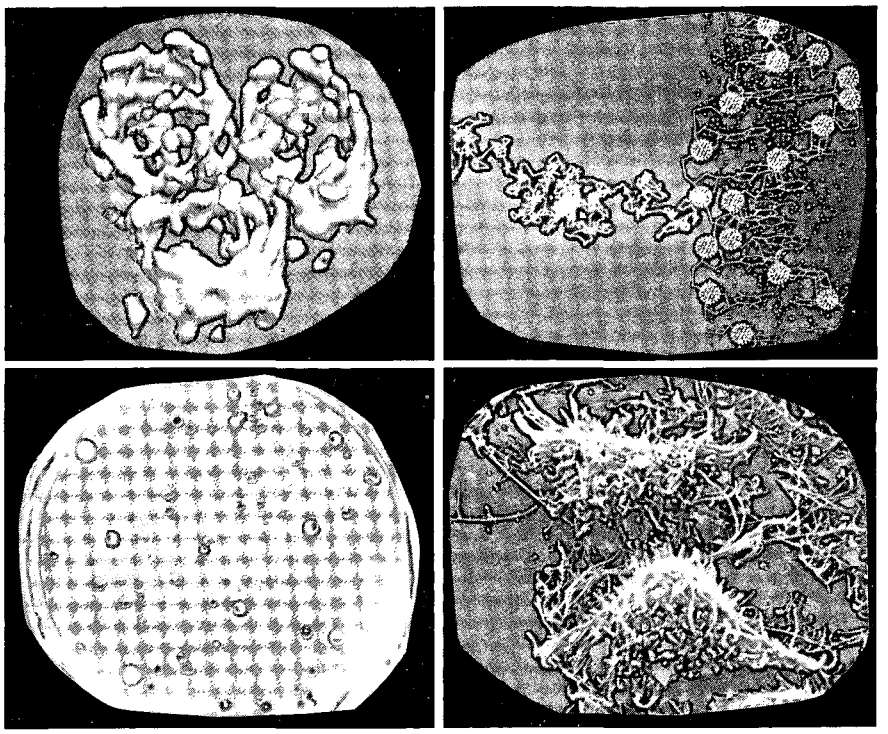

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PROTEIN-DIRECTED MODULATION OF HIGH-LET HYPERTHERMIC RADIOSENSITIZATION

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Protein-directed modulation of high-LET hyperthermic radiosensitization

by

Polly Y. Chang

ABSTRACT

A pair of Chinese Hamster Ovary cell lines, the wild-type CHO-SC1, and its temperature-sensitive mutant (CHO-tsH1) was used to examine the importance of protein synthesis in the development of thermotolerance. The classical biphasic thermotolerant survival response to hyperthermia was observed in the SC1 cells after continuous heating at 41.5°C to 42.5°C, while tsH1 showed no thermotolerance. In separate experiments, each cell line was triggered and challenged at 45°C. The heat doses were separated with graded incubation periods at 35°C or 40°C for thermotolerance development. SC1 cells expressed thermoresistance, with the synthesis of heat shock proteins, under both incubation conditions. tsH1 cells expressed thermotolerance similar to that seen in the SC1 cells when incubated at 35°C, but the survival response with the non-permissive 40°C incubation was much reduced in the absence of protein synthesis. It is therefore postulated that the synthesis of new cytosol proteins is not required for the initial onset of thermotolerance but is necessary for the sustenance of tolerance.

The combined effects of heavy-ion radiation and hyperthermia were examined using the same cell system. A mild heat dose of 41.5°C was used in conjunction with Neon particle radiation of various high LET values. The cell killing effects were highly dependent on the sequence of application of heat and Neon radiation. Heat applied immediately after Neon irradiation was more cytotoxic to SC1 cells than when heat was applied prior to the

irradiation. The ability of cells to synthesize new proteins plays a key role in this sequence-dependent thermal radiosensitization. In the absence of protein synthesis in the tsH1 cells, the high-LET thermal enhancement for cell-killing was unchanged regardless of the sequence. In the presence of protein synthetic activity in the SC1 cells, the thermal enhancement of radiation-induced cell killing was LET-dependent. Furthermore, the RBE of heated-SC1 cells varied with the LET value and reached a maximum of >3, while a reduced maximum RBE of 2.6 was obtained for the heated-tsH1 cells. These results suggest that high-LET radiation induced cellular damage can be potentiated with heat-induced new protein synthesis during post-irradiation heat treatment.

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INTRODUCTION

Overview

The biological effects of densely ionizating radiation have been studied since the early 1930s. The rationale for the use of particle baems with high linear energy transfer (high-LET) radiation for cancer therapy has been based on the considerations of favourable depth-dose distribution, higher relative biological effectiveness (RBE) and reduced oxygen enhancement ratio (OER) (Blakely et al., 1984) compared to low LET radiation such as x-rays or γ rays. Clinical trials are now in progress utilizing these characteristics of helium and neon beams to assess the validity of using heavy-ion radiotherapy in the treatment of cancer (Castro et al., 1985). Protein synthetic activity has been shown to be important for the repair of x-ray induced lesions (Yezzi et al., 1988), however, the relationship between protein synthetic activities and the repair of heavy-ion induced radiolesions has not been established. In addition, it is generally accepted that the damage resulting from particle radiation is not modifiable by hyperthermia or x-rays. Basic laboratory research in the molecular and cellular effects on living cells is continuing, particularly for the purpose of revealing the basic mechanisms of action of these unique radiations on DNA and other relevant molecules.

The biology of hyperthermia is another area of intense research and recent advances include not only clinical trials to assess the feasibility of using hyperthermia as a modality in cancer therapy, but also insight into the molecular mechanisms leading to the regulation of hyperthermic response in various cellular systems. The rationale for the use of hyperthermia in cancer therapy has been based on the fact that heat selectively kills cells in S phase

(Dewey 1977) and that the toxicity of heat is pH-dependent (Hahn 1982). Tumors with actively dividing cells and low pH regions are thought to be candidates for preferential sensitivity to heat. Protein synthesis, particularly heat shock protein synthesis, is thought to be involved in the response to hyperthermia.

Protein synthesis has long been implicated in the development of the cellular response to thermal stress and radiation repair processes. The phenomenon of thermotolerance, whereby heat shocked cells can develop a resistance to a subsequent heat treatment, has led to studies on protein synthetic activities. Many previous attempts to examine the role of protein synthesis in the development of thermotolerance have involved the use of inhibitory drugs, such as cycloheximide or puromycin, to block protein synthesis. Cells that were perturbed by these drugs respond with decreased sensitivity when exposed to x-rays or hyperthermia (Henle and Leeper, 1982; Lee and Dewey, 1986; Lee et al., 1987). Concentrations of cycloheximide from 2.5 mg/ml to as high as 100 mg/ml have been used (Henle and Leeper, 1982; Hallberg 1986; Kraus et al., 1986; Lee and Dewey, 1986; Przybytkowski et al., 1986; Freeman et al., 1987). It has also been demonstrated that the temporal relationship between heat treatment and inhibitory drug administration is important, for reasons that are as yet unknown. Cycloheximide has been shown to be more than an inhibitor of protein synthesis. It is known that it affects glucose transport and metabolism, lipid metabolism (Jomain-Baum and Hanson, 1975; Kalckar et al., 1980), DNA synthesis (Highfield and Dewey, 1972), the level of charged tRNA (Martin 1980), and stimulates actin gene transcription in concentrations as low as 1 mg/ml by inhibiting the peptidyl

transferase activity of the 60S ribosomal subunit (Elder et al.,1984). The use of this drug, therefore, complicates the interpretation of experimental results.

In order to examine this question of protein synthesis involvement in hyperthermic and radiation responses, a pair of cell lines, CHO-SC1 and its temperature-sensitive mutant, CHO-tsH1, have been selected. The temperature sensitive mutant, tsH1, has a structural alteration in the cytoplasmic non-mitochondrial leucyl-tRNA synthetase (leu-RS) such that at non-permissive temperatures of 40°C and above, under normal growth culturing conditions, the rate of protein synthesis is decreased to final levels of 2% of control in less than 1 hour while the wild-type SC1 cells, under the same conditions, continue to synthesize proteins. The growth characteristics for these cell lines are virtually identical and the inhibition of protein synthesis in these tsH1 cells is rapidly reversible by simply altering the culturing temperature. This cell system is, therefore, ideal for studying the relative importance of active protein synthesis in thermal effects alone, and in thermal radiosensitization without the interference of side effects of drugs.

The combined effects of low-LET radiation and heat have been studied and are known to be synergistic (Hahn 1982). Gerner and Leith studied the combined effects of high-LET radiation and heat in the late 70s by applying heat to CHO cells before irradiating with particle beams (Gerner et al., 1976, 1977). The results presented show mere additivity between the two modalities of treatment. Animal studies using neutrons combined with hyperthermia also show no added advantage in local tumor control (Hahn 1976). Since then, very little data is available in this area.

Objectives

The objectives of this research were to: (1) characterize the survival response of the CHO-SC1 and CHO-tsH1 cells to hyperthermia; (2) examine the involvement of active protein synthesis in the hyperthermic sensitivities of these cells; (3) test the effectiveness of the interaction of heavy-ion radiation and hyperthermia in the modification of cellular survival, specifically, the sequence of application of the two modalities; and (4) examine the involvement of heat-induced protein synthesis in the modification of the cellular response to high-LET radiation.

BACKGROUND

Biology of hyperthermia

Hyperthermia kills cells in a stochastic manner. The quantitation of heat dose depends on the temperature of exposure, and on the duration of heat. There is increasing evidence suggesting that the critical primary targets for heat damage are the cellular membranes (Borrelli et al., 1986), and cytoskeleton and nuclear matrix elements (Warters et al., 1987; Dewey 1989), resulting in secondary effects including the loss of ability to maintain intracellular ion gradients (Vidair and Dewey, 1986), changes in intracellular glutathione (Freeman et al., 1990) and ATP levels (Kjellen et al., 1986; Fellenz and Gerweck 1988). Normal protein synthesis is altered by heat, and is accompanied by the disruption of polysomes (Henle and Leeper 1982; Warters and Stone 1983).

Cells at different phases of the cell cycle have different sensitivities to heat, with S-phase cells being most heat sensitive (Dewey et al., 1990). The retardation of DNA chain elongation leads to increased sister chromatid exchanges and chromosomal aberrations which have been associated with killing of S-phase cells (Wong and Dewey, 1982; Wong, et al., 1989). Chromosomal aberrations are not seen in heated G_1 cells. However, cell kinetic studies following hyperthermia reveal that both S and G_1 cells undergo division delays of as long as 12 hours post-heat treatment. This is contrasted with the radiation-induced division delay which is more cell cycle specific (Dewey et al., 1990).

Mammalian cells respond to the milder elevation of surrounding temperature to 43°C or below by eliciting a number of changes that result in a transient resistance to additional heat dose (Ashburner and Bonner, 1979; Nover 1984; Lindquist 1986; Morimoto et al., 1990). Such a response is called the 'heat shock response'. The resistance to further heat damage is termed 'thermotolerance' and generally a specific group of proteins, the 'heat shock proteins' (hsps) are synthesized (Henle and Leeper, 1979; Li and Werb, 1982; Velazquez and Lindquist, 1984). Cells exposed to the protein synthesis inhibitory drug, cycloheximide (CHM), are generally found to be more heat resistant than untreated cells. This enhanced tolerance is postulated to be due to enhanced synthesis of heat inducible hsps (Lee et al., 1987). The major heat shock proteins are grouped into families based on their molecular weights (MW) (See Morimoto et al. 1990, for comprehensive review of stress proteins). The synthesis of heat shock proteins (hsps), especially that with MW 70kD (hsp70), can also be induced by other environmental stress, such as serum stimulation. However, the genetic domain for the regulation of this response was found to be distinct from that of the heat-stimulated expression of hsp70 (Lee et al., 1990; Wu and Morimoto, 1985; Wu et al., 1986). This suggests that thermotolerance expression resulting from stimulation by various other stresses may not necessarily be regulated by the same genetic control. The level of thermotolerance that develops is dependent on the duration and the degree of the temperature treatment. Some evidence exists that targets hsps, especially hsp70, as being important to thermoresistance observed in cells in culture (Li and Werb, 1982, Riabowol et al., 1988).

Much of the early work on the effect of heat on gene expression was done using the *Drosophila* model system (Ashburner 1970), where expanded

regions, 'chromosome puffs', were found to be induced in the giant salivary gland chromosomes of these insect larvae after a short 30 min exposure to elevated temperatures. It was later found that these puffs represent induced genes in salivary gland chromosomes and approximately seven new protein species were made in response to heat shock. The universality of the heat shock response became more apparent in the 1970s when the similar responses to heat was reported in chicken embryo fibroblasts (Kelley and Schlesinger, 1978), *E. coli* (Lemaux et al., 1978), and yeast (McAlister and Finkelstein, 1980). Although the response to heat is very similar in all the organisms examined, from bacteria to man, there are distinct differences in the number of proteins induced and the specific molecular weights of these proteins. DNA sequence comparisons of the major heat shock genes in *Drosophila*, yeast and other eukaryotic organisms, have confirmed that these proteins are among the most highly conserved proteins in nature (Bardwell and Craig, 1984; Bardwell and Craig, 1987; Hunt and Morimoto, 1985; O'Malley et al., 1985).

The functions of most hsps appear to be involved in protein metabolism within the cells. They bind to polypeptides, transport these across membranes to their intracellular destinations, and play a role in the folding of these nascent polypeptides into their proper configurations (Flynn et al., 1989; Pelham 1989; Rothman 1989; Beckmann et al., 1990) The functions of major hsps have been studied extensively in bacterial, yeast and *Drosophila* systems, however, only the relevant information pertaining to mammalian cells, particularly rodent cells, will be discussed in this thesis. The most studied heat shock protein, hsp70, consists of two major forms, the constitutive and inducible forms, each comprised of multiple isoelectric

forms. The expression of the constitutive hsp70 appear to be cell-cycle regulated (Milarski and Morimoto, 1986). Mammalian hsp90 also has multiple isoforms and its function appears to be associated with steroid hormone receptors (Catelli et al., 1985; Ramachandran et al., 1988; Catelli et al., 1989; Shyamala et al., 1989) and protein kinase activities. The function of the small ubiquitous hsp, ubiquitin, has been found to be associated with catabolism of damaged proteins (Henshko 1988). The functions of some other hsps are yet unknown.

When cells are stimulated by heat, there is a directional translocation of proteins (Chu et al., 1990; Kampinga et al., 1989), specifically hsp70 (Riabowol et al., 1988; Hayashi et al., 1991), from the cytosol into the nucleus, resulting in accumulation in the nucleoli. Heated CHO cells show an increase in nonhistone proteins isolated with DNA, alterations in chromosomal proteins (Tomasovic et al., 1978), and proteins bound to the nucleoskeleton comprising the nuclear matrix, nuclear envelope and its associated DNA (Wheeler and Warters, 1982). This accumulation of DNA associated proteins is found to be related to heat duration (Tomasovic et al., 1978). DNA processing at the nuclear matrix is inhibited after hyperthermia (Warters 1988) due to the increased protein mass of chromatin (Warters and Stone, 1983; Warters et al., 1987; Warters 1988). When cells are returned to the permissive temperature for growth after heat shock, the excess nuclear proteins are removed from the nucleus followed by the resumption of cell division cycle (Roti Roti et al., 1986). Fluctuations in DNA polymerase β activity correlate with thermotolerance and thermal cell toxicity effect in CHO cells (Mivechi and Dewey, 1984; Dewey and Esch, 1982).

Heavy-ion radiobiology

Heavy-ion radiation is biologically more effective in producing cellular damage than are x- or γ -rays, ie. they have a greater relative biological effectiveness (RBE) (Todd, 1967; Barendsen, 1968). The values of RBE examined in various mammalian systems have been shown to depend on the average energy of deposition of the heavy-ion radiation, or linear energy transfer (LET) as well as on the particle charge and velocity that generate a particular LET value. As the LET of a particle beam increases, the RBE of most repair proficient mammalian cells in vitro increases and peaks at an LET value of approximately 100 - 120 keV/μm. However, with further increases in LET, the relative effectiveness of a particle beam decreases due to what have been termed 'overkill' effects. Plots of inactivation cross-section of V79 cells vs LET from different energies of ion beams from helium to uranium reveal the importance of track structure effects at very high-LETs in cellular survival studies (Wulf et al., 1985). Other main features of the response to high-LET radiation (Barendsen et al., 1966; Pirruccello and Tobias, 1980; Tobias et al., 1982; Blakely et al., 1984; Blakely and Edington, 1985) include the reduced resistance of hypoxic cells resulting in a low oxygen enhancement ratio (OER) compared to x- or γ-rays, reduced split-dose recovery capacity, and decreased variations of radiosensitivity during the cell division cycle (Blakely et al., 1989). Numerous mathematical models have been developed in an attempt to equate radiation-induced DNA damage with cell survival (Kellerer and Rossi, 1978, Tobias 1985; Curtis 1986; Brenner 1990).

The initial damage caused by charged particle radiation is thought to include a variety of DNA lesions, such as single- and double-strand breaks, DNA-protein crosslinks and base and/or sugar alterations. The initial yield of

double-strand breaks are more extensive and the distribution is more complex than that resulting from low-LET radiation (Goodwin 1988). It has been shown that there is a correlation between high-LET radiation induced double-strand breaks and cell death (Ritter et al., 1977), and high-LET induced chromosomal abnormalities and mutation (Roots et al., 1985; Roots et al., 1990).

Roots et al. (1979) reported that the cellular damages induced by particle radiation is less repairable than that induced by low-LET radiation. However, Blakely et al. (1985) showed that, for synchronous human T-1 cells, the capacity of potentially lethal damage repair (PLDR) is dependent on specific phases within the G_1 stage of the cell cycle. Late G_1-cells are more efficient in repairing heavy-ion-induced PLD than early G_1-cells. The probability of misrepair of DNA lesions increases as LET increases (Tobias et al., 1984). Although it is generally believed that charged-particle-induced cellular damage is less repairable, cell survival studies after a rapid sequential exposure to neon particle radiation and x-rays show that there can be interactions between these two modalities and that the damage induced by neon radiation can be further potentiated by x-rays (Ngo et al., 1981).

Yezzi et al (1988), using the same CHO-SC1 and CHO-tsH1 cell systems, have reported that protein synthesis is needed for repair of x-ray-induced-cellular damage. To date, the effects of protein synthesis on heavy-ion radiation induced damage and repair are not well understood. Weber et al. (1990) reported that, in diploid yeast, the level of rRNA synthesis post-irradiation is LET-dependent. Calculations of the inactivation cross-section by very heavy ions (eg. uranium with LETs up to 13,700 keV/μm) suggest that

the target for impairment of rRNA synthesis may involve structures larger than the genes encoding rRNA.

Low-LET hyperthermic radiosensitization

The synergistic effects of combined exposure to hyperthermia and x-rays or ^{60}Co γ-rays have been well documented (see review by Dewey 1989; Dewey et al., 1977; Sapareto et al., 1978; Hahn 1982). The increased sensitization observed in cell survival studies are manifested by a decrease in the shoulder and an increase in the final slope of the survival curves. An increased level of initial radiation-induced DNA strand breaks, and DNA-protein crosslinks has been observed (Warters and Roti Roti, 1982), and a decrease in the repair of double-strand breaks resulting in an increase in the level of residual breaks was found in cells treated with heat and x-rays (Mills and Meyn, 1981; Dikomey 1982). This may be explained by the inefficiency of repair of damaged DNA due to the heat-induced changes in the nuclear structure resulting from the excess protein associated with the nucleus and the nucleoid, masking the DNA damage (Kampinga et al., 1988). Radiosensitive Ataxia telangiectasia (AT) cells, which are thought to be deficient in repair of ionizing-radiation-induced damage have been used in several studies to evaluate the modification of repair of radiation damage by heat. With heat alone, AT cells show the same thermosensitivities and abilities to acquire thermotolerance as the normal human fibroblast cell lines. Thermal enhancement of radiosensitivity was observed in both homozygous and heterozygous AT cells, but the differences in the magnitude of this enhancement are debatable. (Mitchel et al., 1984; Raaphorst and Azzam, 1984).

These results suggest that there may be more than one radiation or thermal repair process, and at least one of these is absent in AT cell lines.

DNA polymerase β has often been thought to be involved in the repair of DNA damage. Measurements of DNA α and β polymerase activities with and without the inhibition of protein synthesis using CHM in CHO cells suggest that polymerase β is an important enzyme involved in repairing x-ray-induced damage that can cause cell lethality (Chu and Dewey, 1987; Mivechi and Dewey, 1985). In contrast, similar studies using HeLa cells suggest that DNA polymerase α and β do not always correlate with the extent of heat radiosensitization (Kampinga et al., 1989). Therefore, heat inactivation of these enzymes may not be taken as a general cause for the synergistic effect of hyperthermia and radiation, and their relative involvement in the repair of DNA damage may be cell-line-dependent

The sequence of applying heat and x-rays appears to be important, and is dependent on the temperature range. In general, hyperthermia prior to x-rays is more effective with high heat dose (temperatures of 43°C or above and longer exposure times). When lower temperatures (43°C and below) are used, especially when the cytotoxic effect of heat is small, hyperthermia following x-rays is more effective.

Although most cells in culture show synergistic effects when heat is used in combination with low-LET radiation, however, there are both qualitative and quantitative differences in the magnitude of these responses. Thus far, no correlation has been found between heat and/or x-ray response and a number of cellular characteristics, such as, cell chromosome number, DNA content, cell volume and cell population doubling time. Taken

collectively, the experimental evidence suggests that caution should be exercised in making generalizations about the interactions of hyperthermia and ionizing radiations (Li and Hendrik, 1977; Raaphorst et al., 1979; Cohen et al., 1988).

High-LET hyperthermic radiosensitization

In investigating the mechanisms of action of hyperthermia and its modification of radiation damage, Gerner et al (1976) and Gerner and Leith (1977) exposed CHO cells to heat at 43°C before irradiating with helium (LET = 1.6 keV/μm) or carbon ions (LET = 30 keV/μm) from the Bevalac at the Lawrence Berkeley Laboratory. In contrast with the synergistic effects of combining low-LET radiation and hyperthermia, the combined effects of high-LET radiation and hyperthermia were found to be merely additive. Hahn et al. (1976) examined the combined effects of neutron and hyperthermia on tumor tissue, Ridgeway osteogenic sarcomas, and found no advantage to local tumor control. Since then, very little progress has been made in this area of research.

It is, therefore, the purpose of this project to further investigate the combined effects of neon particle radiation and low temperature hyperthermia and examine the involvement of active protein synthesis in the interaction between the two modalities of treatment.

MATERIALS AND METHODS

Cell Culture

1. Cell line

CHO-SC1 and CHO-tsH1 cells were obtained from L. Thompson at Lawrence Livermore National Lab. (LLNL) (Thompson et al., 1973; Haars et al., 1976). CHO-tsH1 cells were isolated with the parent CHO-SC1 cell, selected with MNNG (N-methyl-N-nitro-n-nitrosoguanidine) and further selected at 38°C in the presence of ^{3}H-thymidine (^{3}H-TdR) such that cells actively synthesizing DNA were selectively killed. The CHO-tsH1 cell has a structural alteration in the leucyl-tRNA synthetase such that the formation of leucyl $tRNA^{leu}$ is defective (Haars 1976). It has been found that these tsH1 cells grew at the non-permissive temperature of 38°C when the external leucine concentration was elevated 30- to 100-fold. Intracellular leucine pool size measurements showed that concentration of leucine increased two-fold with any increase in temperature compared to that of the wild type cell. This led to the conclusion that intracellular leucine concentration is involved as the regulatory response to the low leucyl-tRNA synthetase activity. (Molnar and Rauth, 1979; Molnar et al., 1979). The main characteristic of this mutant cell line is that, under non-permissive temperature conditions, the cytosol, non-mitochondrial protein synthesis is inhibited to less than 10% of the control level. Anderson et al (1979; 1980; 1984) studied nuclear protein synthesis using this cell line and proposed that the nuclei contain a leucyl-tRNA synthetase able to function at 40°C and that there is residual synthesis of acidic nuclear protein in the presence of cycloheximide (CHM). These nuclear proteins labeled under non-permissive conditions were speculated to include

some of the "prompt" heat shock proteins (Reiter and Penman, 1983). Experimental techniques used by Anderson et al. (Anderson and Baranowski, 1984, Anderson et al., 1979) however, do not preclude the prompt translocation of proteins from the cytosol into the nuclear fraction. There is, as yet, no firm evidence for the existence of protein synthesis within the nucleus, and further research in this area is necessary.

2. Growth Conditions

Both cell lines were grown in Minimum Essential Medium (MEM) with Earle's salts supplemented with 10% Fetal Bovine Serum (GIBCO), 0.31 mM MEM non-essential amino acids (GIBCO), 0.25 mg/ml L-Glutamine (GIBCO), 1.94 mg/ml $NaHCO_3$, 43 units/ml potassium penicillin G, and 43 mg/ml streptomycin sulfate (Microbiological Associates). Cultures were grown at the permissive temperature of 35°C in a humidified incubator supplemented with 95% air, 5% CO_2. The doubling times for both cells are quite similar and range between 17 - 20 hrs, with the tsH1 cells growing slower by 0.5 - 1 hr. Plating efficiencies for both of these cells range from 80% - 100%. These cells tend to be less firmly attached than most other monolayer cells in culturen since these cells were initially selected to be grown in either monolayer or suspension. For colony formation, large colonies tend to form tails (microcolonies around the parent colony) which may lead to some uncertainty in the counting of colonies.

For experiments, cells were generally seeded at a density of 2×10^5 cells per 25 cm^2 Costar flasks, prefilled with 5 mls of medium, 2 days in advance. The plated cells were maintained in exponential growth. After heat and/or radiation treatments, cells were usually trypsinized and plated for colony

formation (Puck and Marcus, 1956). Briefly, the cell monolayer was rinsed with 1 ml of 0.25% trypsin without EDTA The cells were then exposed to 1 - 2 mls of trypsin at room temperature. The detached cells were neutralized with 4 mls of medium and a 1-ml aliquot of cells was added to 9 mls of isotone in a vial for Coulter counting. After determination of the cell concentration in the initial volume, serial dilutions were carried out such that the appropriate number of cells was seeded into each flasks to yield 100 - 200 colonies per 25 cm^2 Falcon flasks. The number of cells seeded was based on an estimate of the number of surviving cells after the heat/radiation treatments. Cells from each sample point were plated into 4 flasks except for those samples where the survival fraction was expected to be lower than 1%. For these, 8 flasks were plated. After seeding the cells into the prepared flasks, these were returned to the 35°C incubator for 7 days for colony formation. After the incubation period, the medium was removed and colonies were fixed and stained for 5 min with 2 mls of 0.1% crystal violet stain made up in 30% ethanol. Colonies with greater than 50 cells were considered survivors.

Hyperthermic treatment

25 cm^2 Costar flasks containing monolayer of cells in exponential growth were completely filled with supplemented MEM at 35°C, pH 7.4. A Braun Thermomix Water Bath with a precision of ± 0.02°C was used for the 41.5°C, 42°C, 42.5°C and 45°C heat treatments. The flasks of cells were completely immersed in the water baths for various times. Immediately after the heat treatment, the medium was removed by suction. Both cell lines were rinsed with 0.25% trypsin without EDTA and trypsinized at room

temperature for 2 min. In order to compare and control the total times of thermal exposure, both cell lines were removed from the water bath, trypsinized and plated at the same time.

The toxic effects of trypsin after hyperthermia were tested in a separate experiment where sample cultures were seeded at clonal density and heated for various times at 42°C. After heat treatment, the hot medium was replaced with 5 mls of warm 35°C medium and incubated for colony formation without post-heat trypsinization.

For the survival studies using both irradiation and heat, cells were treated with both agents simultaneously with the maximum lag time between radiation and heat being less than 5 min.

Irradiation

150 kVp x-rays at 10 mA (with filtration of 0.5 mm Cu and 1 mm Al) was used as the reference radiation. The samples were irradiated with the beam directed downwards with typical target distance being 20 cm. Dosimetry was done for each experiment using the Victoreen R-meter. Air density correction was obtained by noting the temperature and pressure reading on the day of the experiment. Typical dose rates were about 1 Gy/min. Typical exposure times were in the order of minutes. The LET of x-rays was estimated to be about 1 keV/μm (Johns and Cunningham, 1974)

Unmodified neon ion (425 MeV/amu) beams from the Bevalac at the Lawrence Berkeley Laboratory with four different LET values from 32 keV/μm in the plateau to 183 keV/μm near the peak position were used in

these studies (Blakely et al., 1979; Ngo et al., 1981) (Figure 1). Dosimetry done for each experiment included the measurement of the Bragg peak. Three ion chambers (ICs) upstream of the sample were used to measure the variations of radial distribution of ionizations along the beam path. The dose IC, sandwiched between the water absorber and the sample flask, was programmed to cut off at the desired dose. A calibrated 1 cm^3 tissue-equivalent thimble ion chamber (EGG model no. IC-17A) was placed immediately after the dose IC to calibrate for the actual delivered dose to the samples before placing the sample flask along the beam line. All radiation exposures were done at room temperature. From the position of the Bragg peak, the residual ranges for the four different LETs used were determined after the range of the Bragg peak was located (Figure 1). Table 1 lists the residual ranges used for the four LETs examined. These Bragg curve measurements were done for each experiment to control for the variability of the heavy-ion beam. The variable water absorber was used to vary the residual range of the beam.

Typical dose rates were adjusted to be about 1 - 2 Gy/min. 10/64 inch lead foil scatterer was used to give a uniform beam spot of > 4 cm in diameter as confirmed by x-ray film. Each sample flask was irradiated in the vertical position through the bottom of the flask and the dose to the cells were corrected for the thickness of plastic upstream of the cell monolayer. No dose correction factor was used for the plateau position and dose correction factors of 1.06 and 1.18 were used for LETs of 100 keV/μm and 183 keV/μm (Pirruccello and Tobias, 1980).

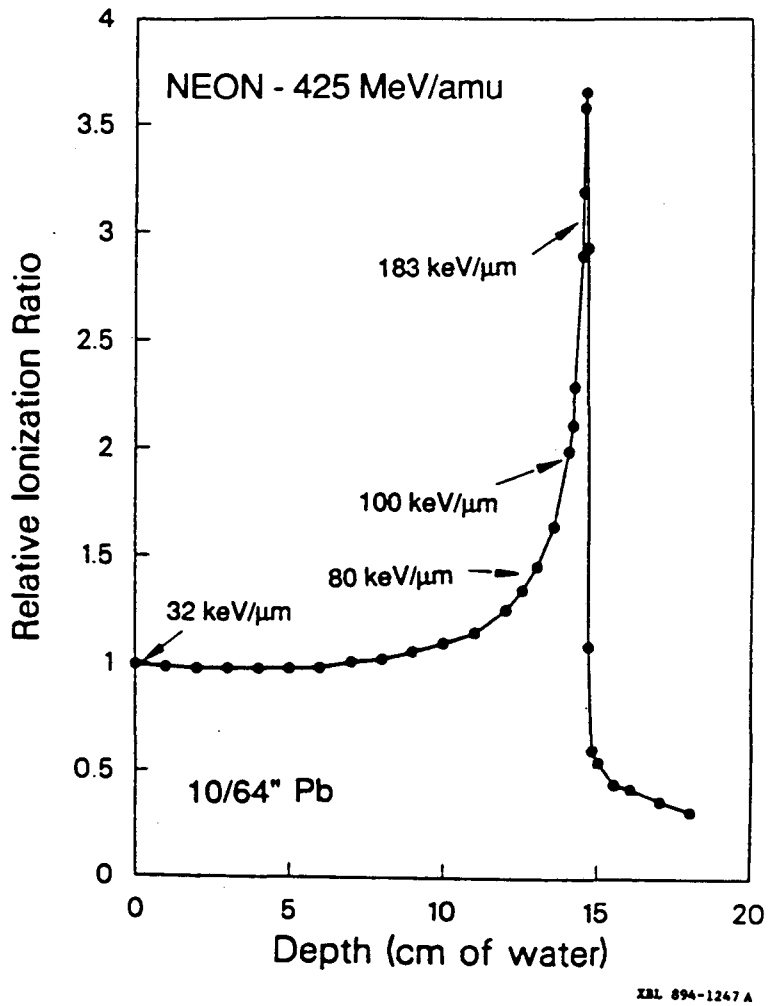

Figure 1 Bragg ionization curve of 425 MeV/amu neon ions showing the four different LET positions used. 10/64 inch lead foil was used as scatterer to enlarge the beam spot to cover the sample flasks.

Table 1 LET values and their residual ranges upstream from the Bragg peak.

LET (keV/μm)	Residual Range (cm of H_2O)
30	full range
80	1.2
100	0.5
183	0.2

Rates of protein synthesis

Cells were heat-treated as was previously described. At various times after heat treatment, 120 µl of ^{3}H-leucine (^{3}H-leu) (1 mCi/ml, specific activity 60 Ci/mmole, New England Nuclear) was added to the samples containing about 25 mls of complete medium, to reach a final concentration of 3 - 5 µCi/ml for an additional 5 min at the elevated temperature. Immediately after the labeling period, the cells were trypsinized, an aliquot taken out for Coulter counting, and the rest of the sample was loaded onto 0.22µ Millipore filters. The cells on the filters were washed twice with ice-cold phosphate buffered saline without Ca^{++} and Mg^{++} (PBS-CMF), twice with ice-cold 5% Trichloroacetic Acid (TCA) to precipitate the proteins, and twice with ice-cold 95% ethanol for fixation. The filters were air dried and counted with a scintillation counter in 10 mls of Instagel (Packard Instruments). Background samples were prepared by loading equal amounts of unlabelled cells onto the Millipore filters and processing them the same way as the labeled samples. All scintillation counts were corrected for cell number.

The percent rate of incorporation of ^{3}H-leu was calculated by dividing the background corrected counts for the heat-treated cells by the background corrected counts for the unheated control cells.

In separate studies, the rate of protein synthesis was measured using ^{35}S-methionine (^{35}S-met) (20 µCi/ml, specific activity at 1200 Ci/mmole, New England Nuclear). The cells were heat-treated or irradiated and then heat treated. At various times after treatment, the medium in the flasks were replaced with 5 mls of medium containing ^{35}S-met at about 20 µCi/ml and pulse-labeled at the appropriate temperature for a period of 30 min. After the

labeling period, the radioactive medium was suctioned off. The samples flasks were rinsed once with cold PBS-CMF and the attached cells were trypsinized at room temperature for 2 min. After neutralization with ice-cold medium containing 10% serum, the cell suspension was collected and centrifuged for 5 min at setting #4 in a clinical tabletop centrifuge. The supernatant was disposed and the cell pellets were resuspended in ice cold PBS and concentrated by centrifugation. This procedure was repeated twice. After the final wash, the cells were resuspended in a total of 1 ml PBS-CMF. A 10 µl aliqout was taken for cell counting, 20 µl was placed in a scintillation vial to determine the rate of uptake of ^{35}S-met, and the rest of the cells were divided into 200 µl fractions and frozen in eppendorf tubes at -70°C for further protein SDS-PAGE analysis. Cells in the scintillation vial were lysed with 1 ml of lysis buffer (see Appendix A) for 2 min. Direct viewing under the microscope for the action of SDS-lysis buffer on monolayer cells confirmed that cells were completely lysed within 10 secs after exposure to this SDS-lysis buffer. 10 mls of Econoflour scintillation fluid was added to each vial and counted in a Packard scintillation counter. The ^{14}C standard provided with the instrument was used for calibration. Upper energy value used for ^{35}S measurements was 168 keV. Each sample was counted for 5 min. Unlabelled cells lysed with the same SDS-lysis buffer in 10 mls of Econoflour were used as the background sample. The rates of protein synthesis were corrected for cell number and normalized to the untreated control similar to the calculation methods done in the ^{3}H-leu studies described in the previous paragraph.

Total protein measurements

The Biorad protein kit was used to microassay for the total amount of proteins in the samples stored at -70°C in Eppendorf tubes previously harvested. These samples were thawed and diluted to within the range of 1-20 μg protein in the final cuvette (50 μl of cells added to 1 ml of water). Diluted samples were sonicated for 15 sec surrounded with ice to keep the samples cold and prevent denaturation (See Appendix B for detailed procedure). Standard curves were established each time using Bovine Serum Albumin (BSA). 0.2 ml of the Biorad dye concentrate was added to the diluted samples and the standards. The absorbance at 595 nm was measured using a Perkin Elmer spectrophotometer within 1 hr after adding the dye concentrate. The calculated protein content was corrected for the number of cells in the sample and the total protein present in each sample was expressed as mg of protein per 10^6 cells.

SDS polyacrylamide gel electrophoresis

Frozen samples in Eppendorf tubes that were previously labeled with ^{35}S-met were thawed slowly in an ice bath. An equal volume of 2x sample lysis buffer (see Appendix A) was added directly to the sample tube and the lysed samples were immediately boiled for 5 min at 100°C to denature the proteins. For one-dimensional SDS-PAGE analysis, 12.5% polyacrylamide slab gel was used, and samples were electrophoresed according to the method of Laemmli (Laemmli 1970) at 25 mA constant current per plate. (see Appendix A for the formulation of solutions and running conditions). Based

on Coulter counts, the volume of cell lysate per lane was corrected such that an equal number of cells were loaded per lane. Molecular weight standards obtained from Sigma Co. were: myosin (205 kD), β-galactosidase (116 kD), phosphorylase b (98 kD), bovine serum albumin (67 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD). Gels were stained using the silver stain method (Sambrook et al., 1989) to view constitutive proteins. The silver staining technique is 100- to 1000-fold more sensitive than staining with conventional Commassie Blue. As little as 0.1-1.0 ng of polypeptide in a single band can be detected by the silver stain procedure.

For fluoroscopy, the gels were first soaked in ENHANCE from Amersheim for 30 min, and dried for about 2 hrs at 80°C onto 3MM Whatman filter paper using a heated vacuum gel drier. The migration distances of the different standard molecular weight markers were marked with ink containing trace amounts of ^{14}C. The dried gel was encased in a Kodak cassette with a piece of 8.5 inch x 11 inch Kodak XR-5 x-ray film in direct contact with the dried gel. The cassette was sealed and clamped to ensure maximum contact of film to gel. This cassette was stored at -70°C for 2 wks as described by Laskey and Mills (Laskey and Mills, 1975). After exposure, the film was developed and fixed, and the negative was scanned with a Biorad video densitometer equipped with the 1-dimensional PAGE gel analysis software for the quantitation of the intensity of protein bands.

Cycloheximide treatments

CHO-SC1 cells were treated with 10 μg/ml cycloheximide for about 2 hrs at 35°C prior to radiation and heat treatments. The cells were exposed to

cycloheximide during neon irradiation followed by 4 hrs at 41.5°C heating. Immediately after treatments, the drug was removed, cells were rinsed twice with normal medium, and plated for colony survival.

Data analysis

A computer program developed by Albright (Albright 1987) was used for the data analysis. Survival data was entered into the computer as colony counts, number of cells plated and the radiation dose given. This information was processed by the sunroutine DSUM to generate fractional survival values for each dose value. To fit the survival curves, two models in the FIT sunroutine was used, namely, the linear-quadratic (LQ) model,

$$\log(S) = y_0 - \alpha D - \beta D^2,$$

and single-hit-multi-target (SHMT) model,

$$\log(S) = y_0 + \log(1 - (1 - e^{-kD})^n),$$

where k is the inactivation constant and n is the extrapolation number.

The calculated parameters (α, β, final slope D_0, shoulder D_q, extrapolation number, n) were used in the RATIO subroutine to generate the ratio of doses to determine relative effects such as Relative Biological Effects (RBE)[1] ,

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[1]RBE = Ratio of final slope of the survival curves for x-rays to the slope of the survival curve of the test system, specifically radiation with heat.

Relative Biological Effectiveness at 10% survival (RBE_{10})[2], Thermal Enhancement Ratios (TER)[3] and Thermal Enhancement Factors (TEF)[4]. Each survival curve was repeated at least twice. Error bars represent the 95% confidence interval.

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[2]RBE_{10}= Ratio of radiation dose at 10% survival for x-rays to that which will produce the same survival level of the test system, specifically radiation with heat.

[3]TER = Ratio of final slopes D_0 of the control radiation survival curve to the final slope of the combined radiation and heated survival curve.

[4]TEF = Ratio of doses at 10% survival of the control radiation survival curve to the final slope of the combined radiation and heated survival curve.

RESULTS

Hyperthermic responses of CHO-SC1 and CHO-tsH1

The thermoresponse survival curves for CHO-SC1 and CHO-tsH1 cells were established by exposing these cells to continuous heating at 41.5°C, 42°C, and 42.5°C. Figure 2 illustrates the changes in cell survival after increased times of incubation at these three elevated temperatures. Wild-type CHO-SC1 developed the biphasic survival curve that is indicative of thermotolerance after 3 hrs of exposure to 41.5°C, 5 hrs of exposure to 42°C, and 4 - 5 hrs of exposure to 42.5°C. The protein synthetic mutant, CHO-tsH1, showed no thermotolerance up to 7 hrs in the elevated temperatures, as evidenced by the exponential decrease in cell survival with increasing times of incubation. Both cells showed exponential decrease in survival when they were chronically heated at 45°C (Figure 3). The differences in the shape of the shoulder suggested that tsH1 cells were initially more resistant to heat killing than SC1 cells with the same temperature treatments (Figures 2 and 3). Colony survival results obtained from heated cells without post-heat trypsinization showed identical survival response curves ruling out the possibility that these effects were due to the toxicity of trypsin after heat treatments.

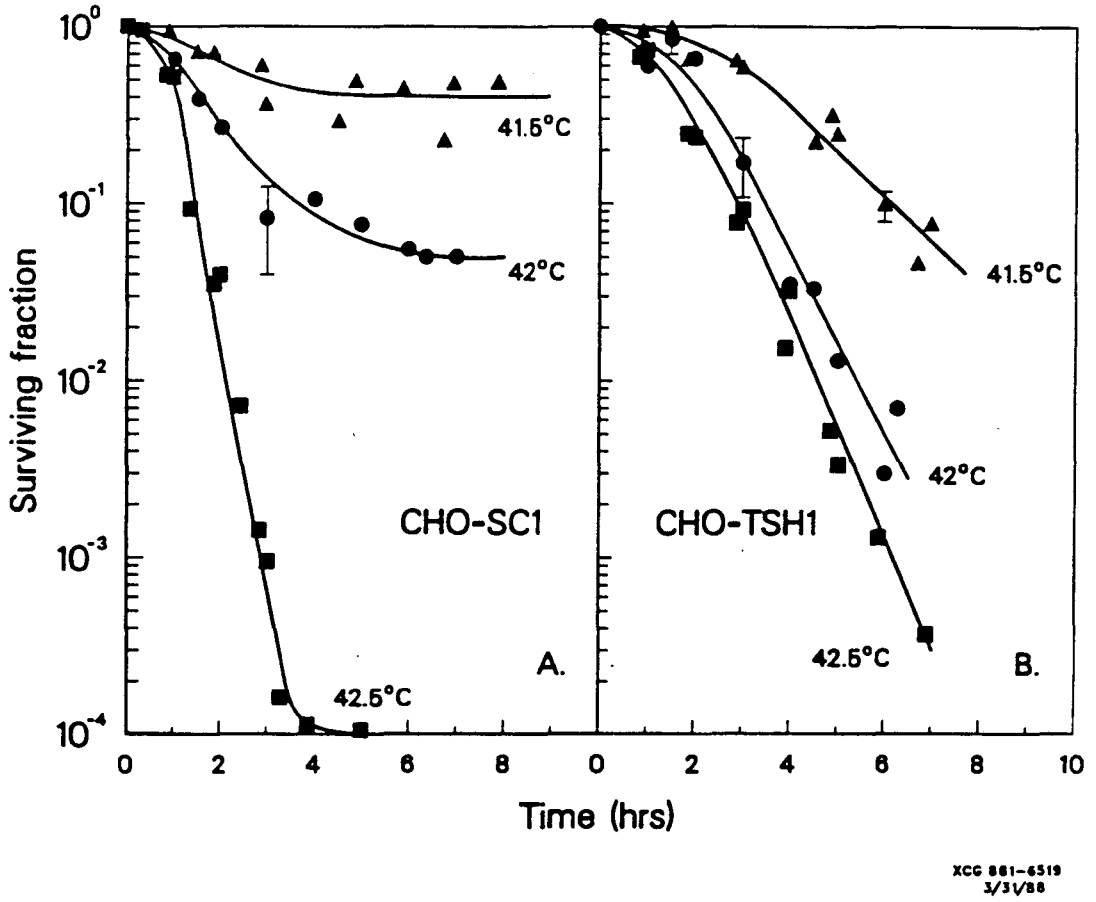

Figure 2 Survival of CHO-SC1 and CHO-tsH1 at 41.5°C (▲), 42°C (●) and 42.5°C (■) as a function of time under continuous exposure to heat. Standard errors are shown when larger than diameter of symbol. Data were obtained from two separate experiments.

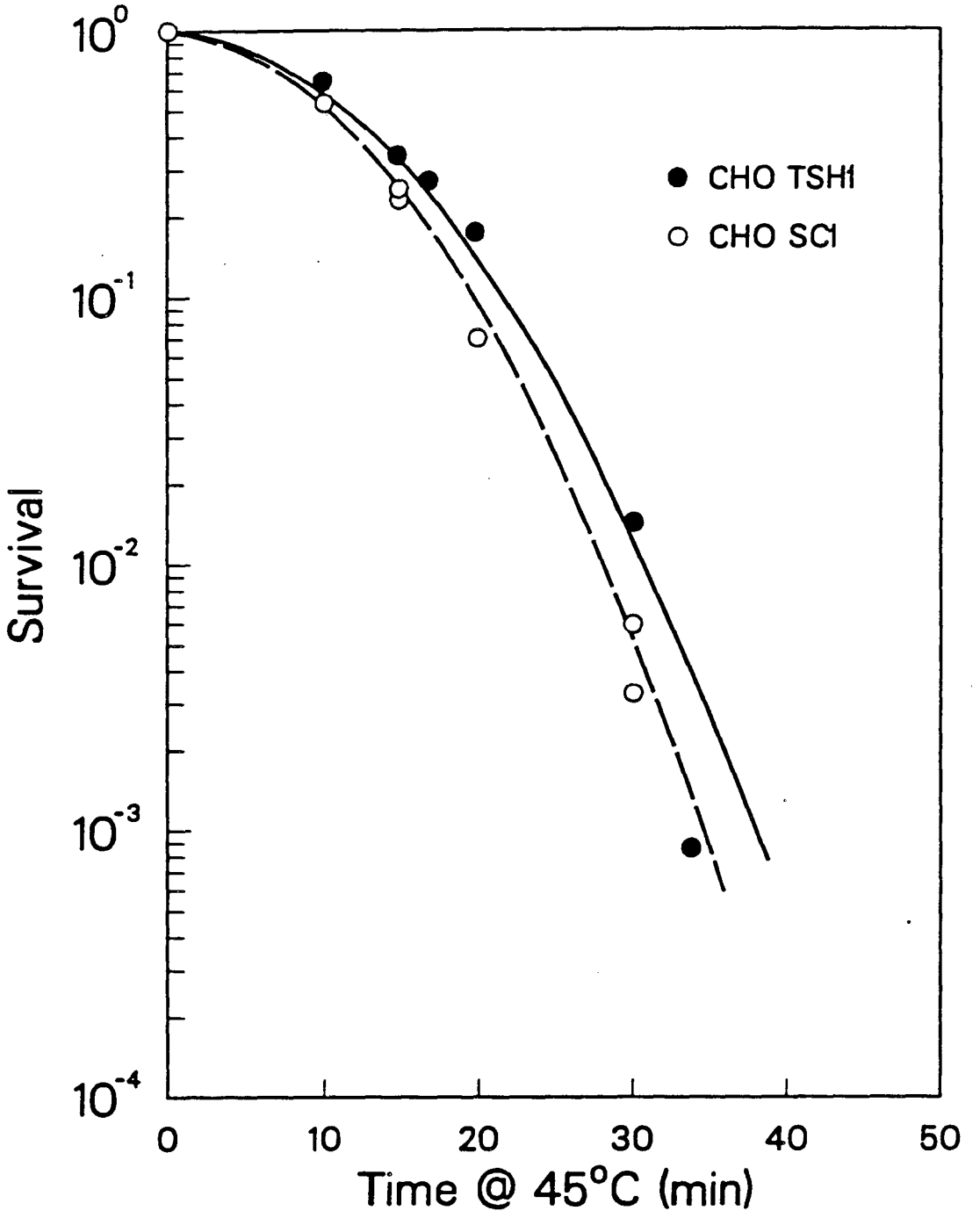

Figure 3 Survival of CHO-SC1 (○) and CHO-tsH1 (●) at 45°C.

SC1 and tsH1 cells were treated with an initial 15 min exposure at 45°C (triggering heat dose) followed by an incubation period at the permissive 35°C for graded intervals up to 24 hrs and then further heat treated with an additional 25 min at 45°C (challenged heat dose). Total 45°C heating time was 40 min (Figure 4) Both cell lines showed an initial very rapid recovery within 1 hr of incubation at 35°C, followed by a slower component, reaching a plateau at a surviving fraction of about 2×10^{-1}. This tolerance was sustained for as long as 24 hrs after the initial triggering temperature dose. No decay of tolerance was observed for the duration of the experiments. The two thermotolerance curves were superimposable. SDS/PAGE autoradiographs (Figure 5) showed that under the above mentioned conditions, both cell lines showed an initial decrease in the uptake of radioactivity compared to the control unheated cells, although the faint but specific bands of heat shock proteins (hsps) are evident. Within 3 hrs of incubation at 35°C, there was preferential increase in the synthesis of heat shock proteins for both cell lines. The major hsps with apparent molecular weights of 110kD, 97kD, 87kD, 70kD, 59kD, 39kD, and 30kD are indicated in the figure.

Total cellular protein of 0.19 ± 0.1 mg per 10^6 SC1 cells and 0.17 ± 0.05 mg per 10^6 tsH1 cells was measured in two separate experiments using the Biorad protein assay kit. Autoradiograms of SDS/PAGE gel for both cell lines indicated that the mutant cells (tsH1) may have a higher rate of uptake of ^{35}S-met than the wild-type cells (SC1). This was evidenced by the differences in the intensity of banding pattern for the same number of cells per lane and the same exposure times (lanes 1 and 4 of Figure 5 and Figure 7).

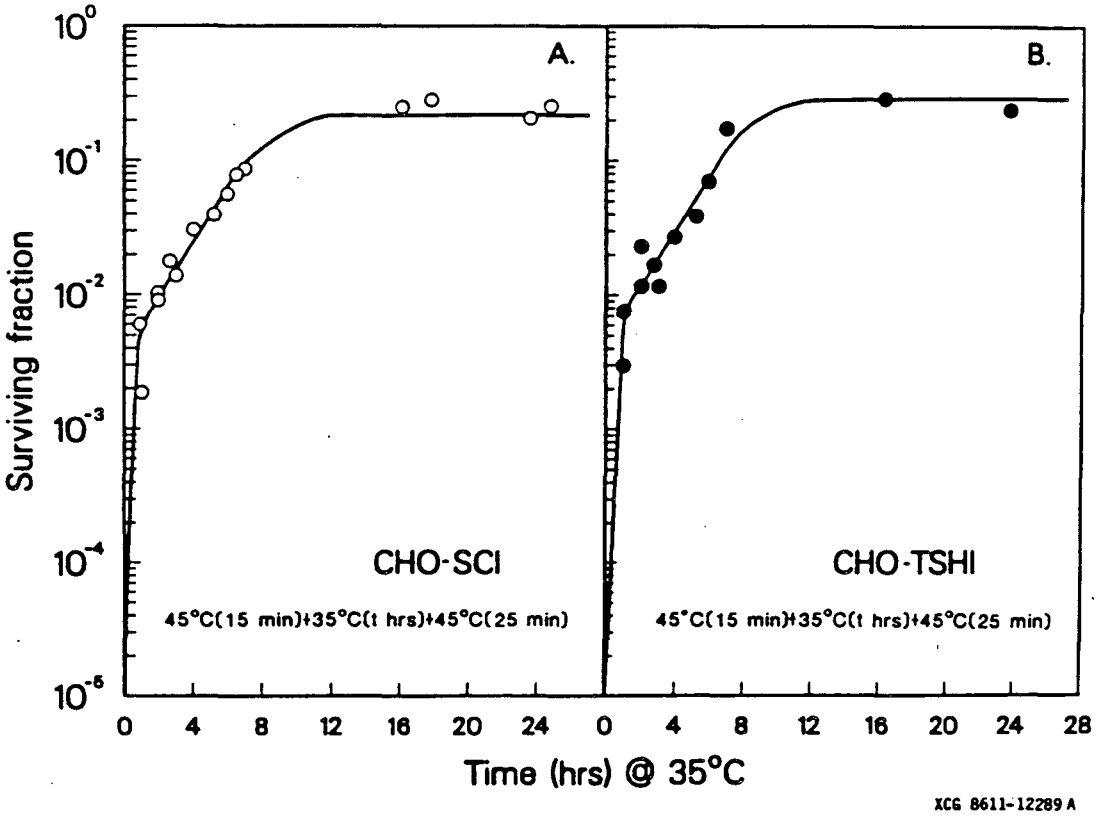

Figure 4 Thermotolerance induced by 15 min at 45°C followed by incubation at 35°C and challenged by 25 min at 45°C for CHO-SC1 (panel A) and for CHO-tsH1 (panel B). Data were obtained from two separate experiments.

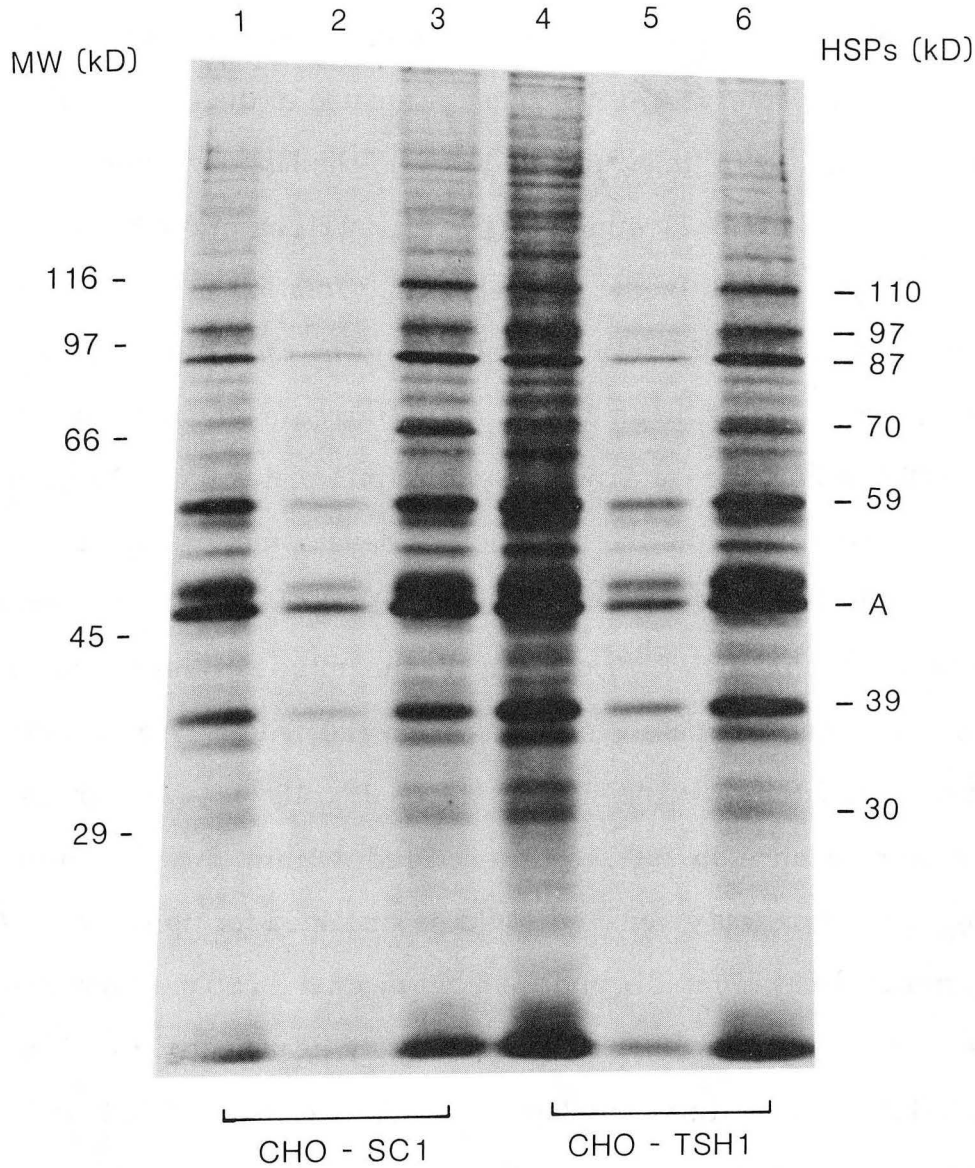

Figure 5 Fluorographs showing protein synthesis after heat shock of CHO-SC1: unheated control 4.3 hr at 35°C (lane 1), heated for 5 min at 45°C followed by 1.3 hr (lane 2) and 4.6 hr (lane 3)at 35°C; and of CHO-tsH1: unheated control 4.3 hr at 35°C (lane 4), heated for 5 min at 45°C followed by 1.3 hr (lane 5) and 4.6 hr (lane 6) at 35°C. Molecular weight markers are shown on the left. Apparent molecular weight of major hsps are listed on the right.

Additional studies were completed where SC1 and tsH1 cells were triggered for 5 min at 45°C, and then incubated at the non-permissive 40°C for graded intervals of times up to 7 hrs. The challenge treatment was 25 min at 45°C for SC1 and 27 min at 45°C for tsH1 in order to attain an initial equivalent survival for the two cell lines. Total 45°C heating times were 30 min for SC1 and 32 min for tsH1 (Figure 6). Wild-type cells showed thermotolerance with an initial rapid increase of survival fraction from 4.5×10^{-3}, followed by a slower rise to a final plateau at 5×10^{-1}, similar to the pattern seen when the thermotolerance development temperature was 35°C. In the tsH1 cells, there was also an initial rapid increase in survival fraction with incubation at 40°C. However, the magnitude of this tolerance was much reduced when compared to that seen in the wild-type SC1 cells under the same conditions (from 5×10^{-3} to 1×10^{-1}). The initial surge of thermoresistance in tsH1 cells occurred within the first hour after the triggering dose was given, and could be sustained for up to 4 hrs. After 4 hrs, tolerance decayed rapidly and by 8 hrs of total incubation time, survival was the same as if the culture was continuously heat-treated for 32 min at 45°C. SDS/PAGE autoradiograms (Figure 7) indicated that SC1 initiated synthesis of heat shock proteins as early as 1.3 hrs after exposure to 40°C and continue at least up to 5 hrs of incubation at 40°C while none, within the limits of the sensitivity of ^{35}S-met labelling technique, was detected in the tsH1 cells as early as 1.3 hrs after incubation at 40°C.

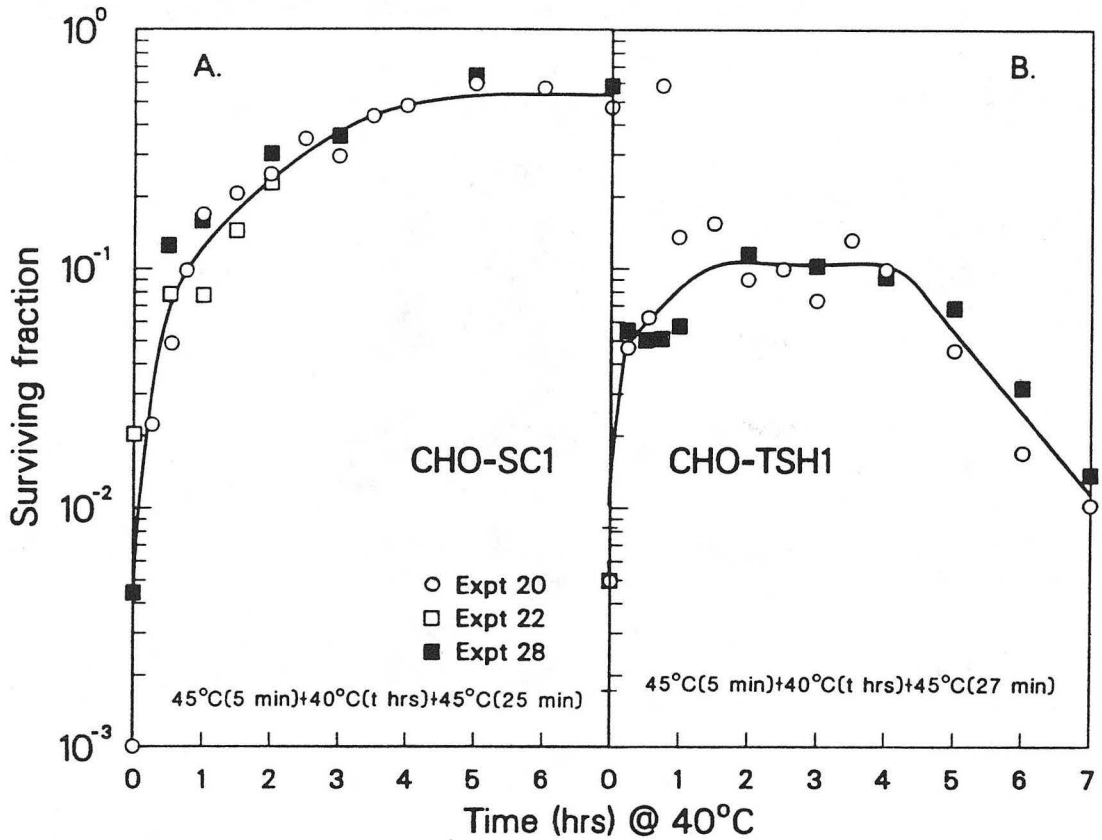

Figure 6 Thermotolerance induced by 5 min at 45°C followed by incubation at 40°C and challenged 25 min at 45°C for CHO-SC1 (A) and 27 min at 45°C for CHO-tsH1 (B). Data presented are pooled measurements from three separate experiments.

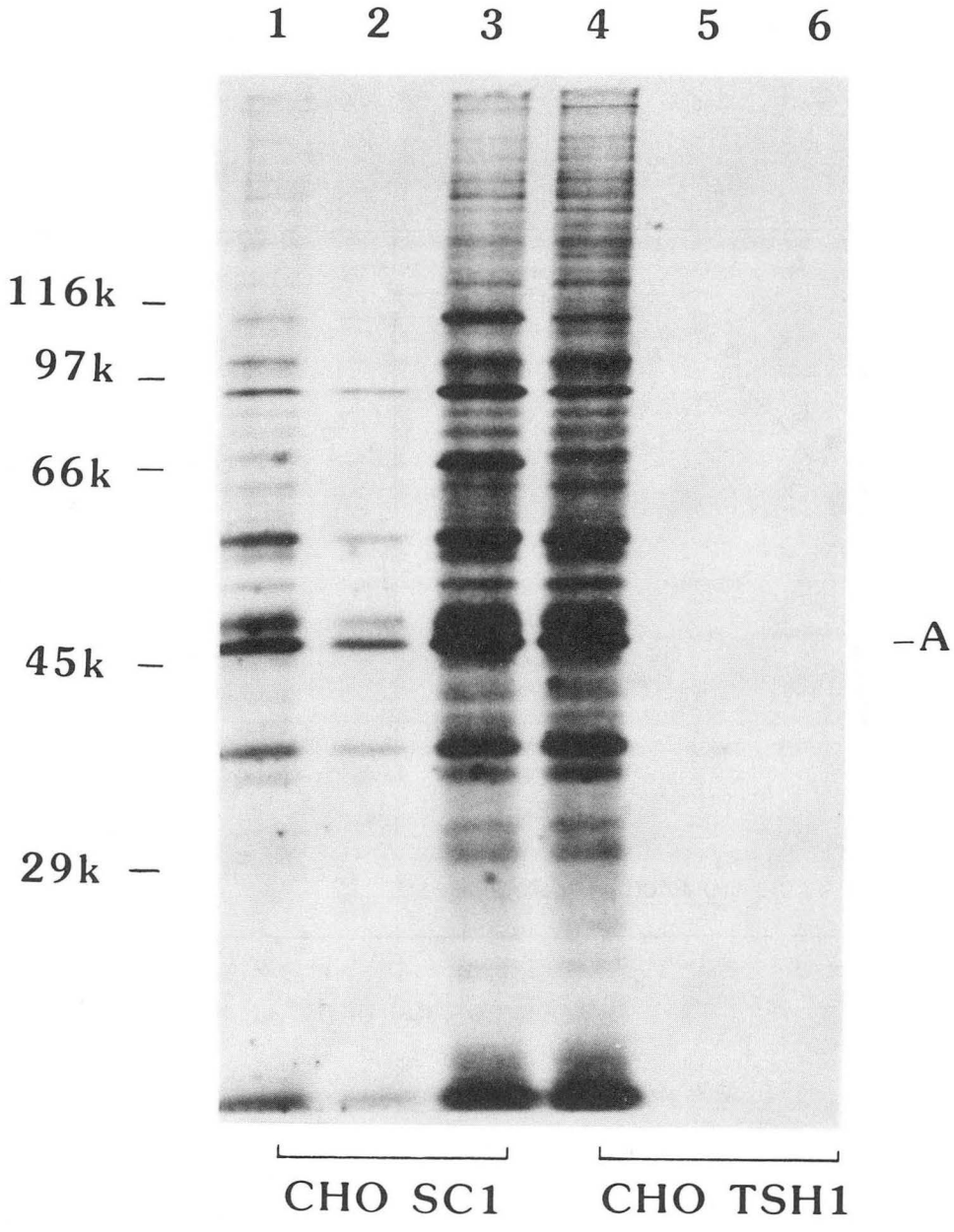

Figure 7 Fluorograph showing protein synthesis after heat shock of CHO-SC1, unheated control 4.3 hr at 35°C (lane 1), heated for 5 min at 45°C followed by 1.3 hr (lane 2) and 4.6 hr (lane 3) at 40°C; and of CHO-tsH1: unheated control 4.3 hr at 35°C (lane 4), heated for 5 min at 45°C followed by 1.3 hr (lane 5) and 4.6 hr (lane 6) at 40°C.

Rates of protein synthesis of CHO-SC1 and CHO-tsH1

Figure 8 shows the changes in the rate of protein synthesis in both the wild-type cells and the temperature sensitive mutant when they were each subjected to continuous 42°C heating from 0 to 8 hrs. Both cell lines show a precipitous drop in the rates of incorporation of ^{3}H-leu within the first hour of heating, but a leveling off at about 30% - 40% of the control level for SC1 and less than 10% for tsH1. Parallel measurements using ^{35}S-met as the labelling isotope-amino-acid complex at 41.5°C showed the same pattern of change in rate of uptake of radioactivity. Although there was a difference in temperature of 0.5°C, within the sensitivity of the measurement technique, the rates are comparable in both cells.

This inhibition of the rates of active protein synthesis was reversible for both cell lines. When CHO-SC1 and CHO-tsH1 cells were heated for 3 hrs at 42°C and then returned to the permissive 35°C, there was an immediate increase in the rates of protein synthesis in both cell types. Although the rate of protein synthesis in the tsH1 cells was more severely inhibited at 42°C than SC1 cells, there was a rapid initial recovery within the first hour. Each cell line took up to 24 hrs to reach the 100% level (Figure 9). Silver-stained SDS-PAGE gels showed that although there were constitutive proteins present in the tsH1 cells, there was no incorporation of radioactivity as demonstrated by the lack of detectable bands in the autoradiograph. However, when these cells were released from hyperthermic treatment, there was uptake of radioactivity and incorporation into the cell to synthesize hsps, as shown by the pattern of bands in the autoradiograph (Figure 10).

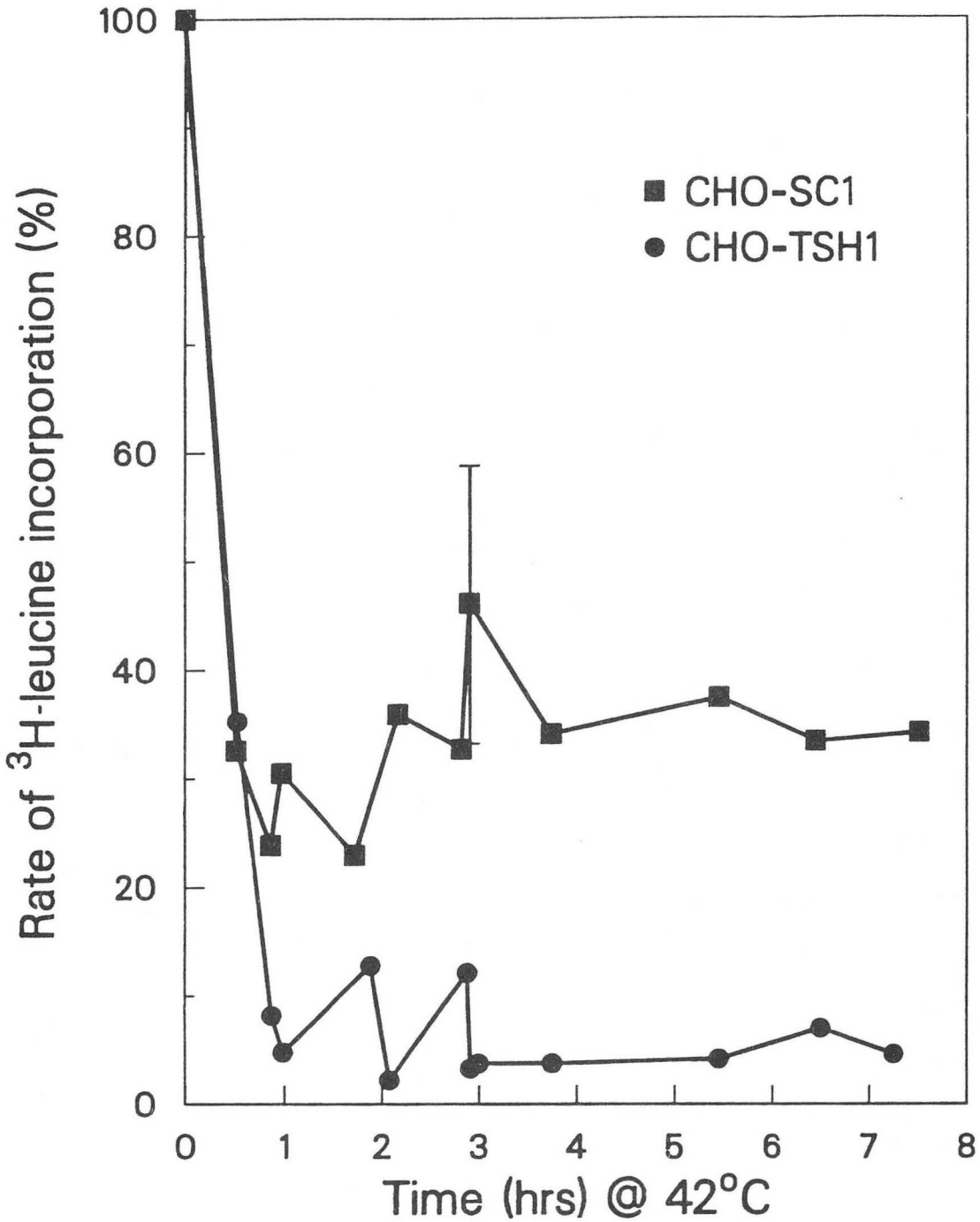

Figure 8 Percent rate of protein synthesis of CHO-SC1 (■) and CHO-tsH1 (●) with continuous exposure at 42°C.

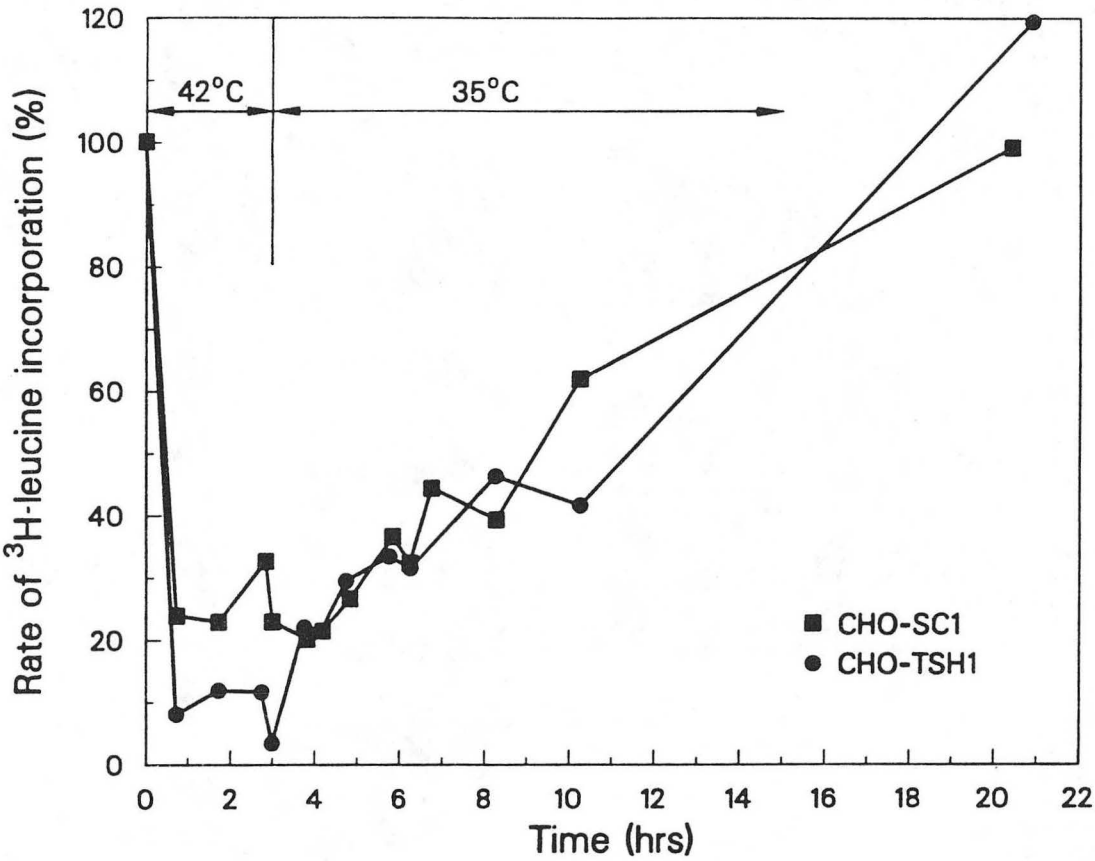

Figure 9 Recovery of rate of protein synthesis of CHO-SC1 (■) and CHO-tsH1 (●) after 3 hrs incubation at 42°C and then returned to the permissive 35°C.

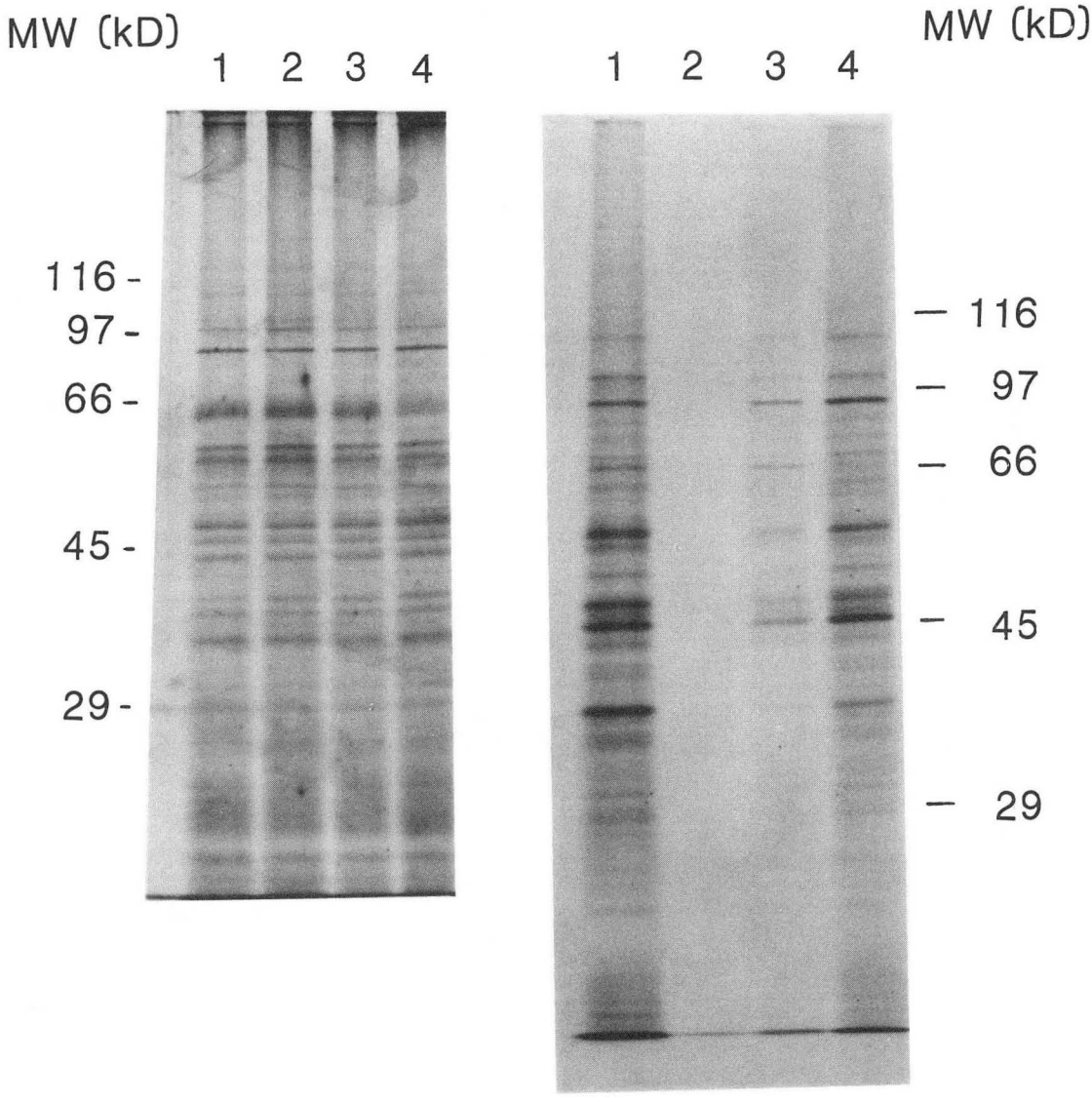

Figure 10 SDS-PAGE gel of tsH1 cells showing silver-stained constitutive proteins (Left panel) and labelled proteins (Right panel) present in the cells. Both panels are derived from the same gel, and equal number of cells were loaded in each lane. Molecular weight markers are shown on either side of the gel photographs. Lane 1 shows control unheated cells, Lane 2 shows tsH1 cells heated for 0.5 hrs at 41.5°C. Lanes 3 and 4 shows the recovery of protein synthesis after 4 hrs at 41.5°C followed by 0.5 and 1.5 hrs at 35°C.

Heat combined with radiation

CHO-SC1 and CHO-tsH1 cells both show the same radiosensitivity to 150 kVp x-rays alone (Figure 11) or 425 MeV/amu neon ions alone (Figures 12 and 13). Table 2 lists the α , β parameters obtained from the linear quadratic (LQ) fit, and n, D_o and D_q values obtained from the single-hit multi-target (SHMT) fit.

Table 2 α and β values generated from LQ fits and n, D_o and D_q values from SHMT fit to the survival data from CHO-SC1 and CHO-tsH1 cells treated with radiation alone.

LET	α (10^{-3})	β (10^{-6})	n	D_q (Gy)	D_o (Gy)
Xrays alone	0.19 ± 0.01	0.06 ± 0.00	5.82 ± 0.69	2.08 ± 0.11	1.18 ± 0.02
30 keV/μm	0.33 ± 0.05	0.06 ± 0.01	3.23 ± 0.85	1.32 ± 0.24	1.13 ± 0.06
80 keV/μm	0.86 ± 0.1	0.02 ± 0.02	1.47 ± 0.27	0.37 ± 0.17	0.97 ± 0.03
100 keV/μm	1.01 ± 0.1	0.01 ± 0.02	1.10 ± 0.2	0.09 ± 0.16	0.93 ± 0.05
183 keV/μm	0.9 ± 0.11	0	1.21 ± 0.23	0.20 ± 0.19	1.03 ± 0.05

Heat treatment, applied singly, for 4 hrs of 41.5°C (Figure 2) is equitoxic to both CHO-SC1 and CHO-tsH1. When SC1 and tsH1 cells were exposed to x-rays, followed immediately by 4 hrs of heating at 41.5°C, the wild type SC1 cells showed increased sensitivity compared to that of the mutant tsH1 cells (Figure 11).

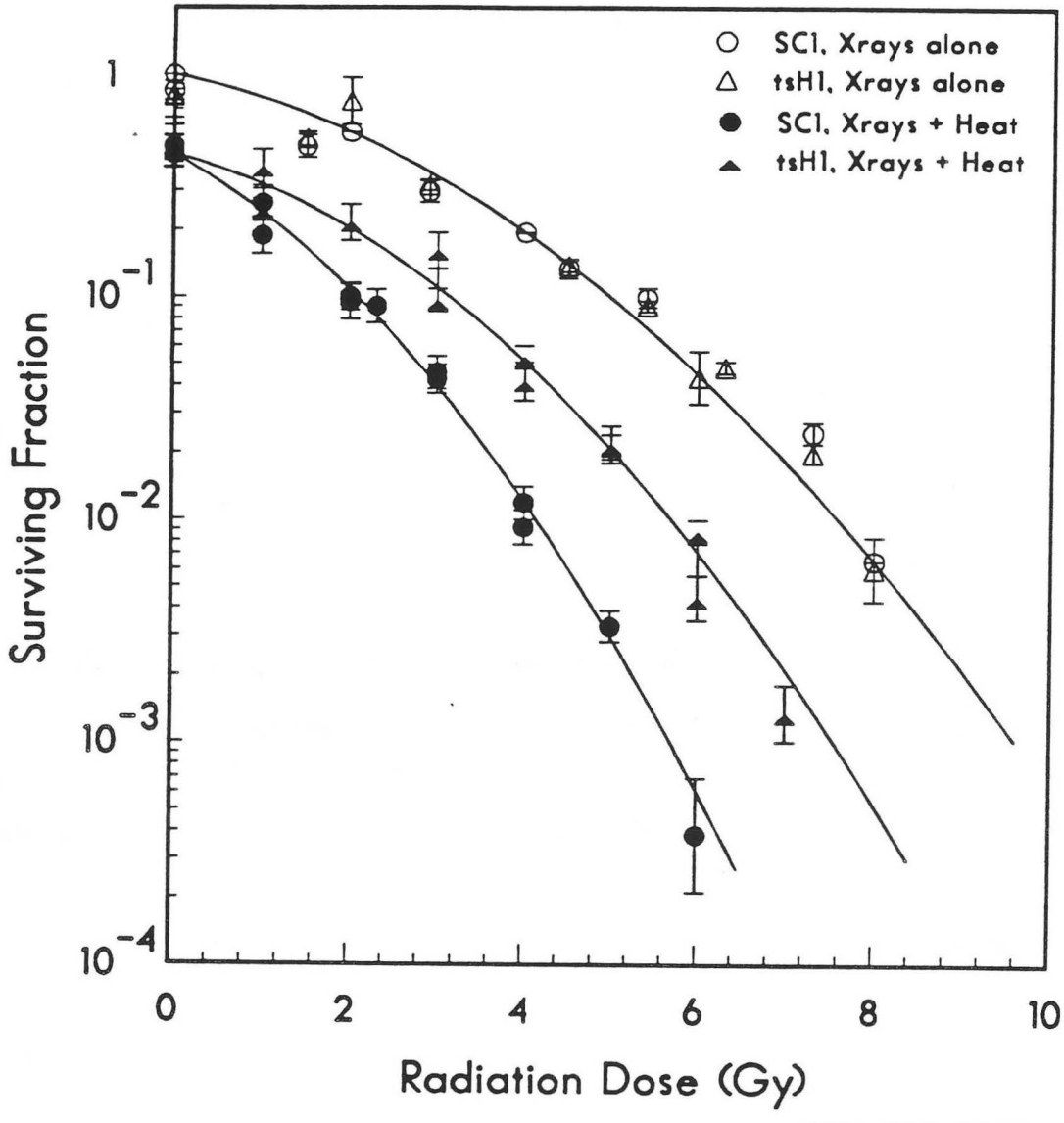

Figure 11 Dose response survival curves of CHO-SC1 and CHO-tsH1 cells after 150 kVp x-rays with or without further heat treatment of 4 hrs at 41.5°C. Both cells have the same sensitivities to x-rays alone. The origins of the heated curves are plotted to reflect the amount of heat toxicity when cells are subjected to heat alone.

When CHO-SC1 cells were preheated for 4 hrs at 41.5°C followed immediately by exposure to graded doses of neon ions with an average LET of 32 keV/μm (Figure 12A), there was a thermal enhancement at 10% survival (TEF_{10}) of 1.50 ± 0.08 compared to that of the unheated cells (Table 3). The quasi-threshold dose (D_q) was reduced from 1.32 ± 0.24 for the unheated curve to 0.67 ± 0.3 for the heated curve, a reduction in the shoulder region of the survival curve (Table 4). The final slope (D_o) was also reduced from 1.13 ± 0.06 for the unheated curve to 0.84 ± 0.08 for the heated curve, giving a thermal enhancement ratio (TER) of 1.26 ± 0.05 suggesting that there was a small enhancement of cell killing when heat was applied prior to heavy-ion irradiation. Using the 150 kVp x-ray survival data as reference, the RBE at 10% survival (RBE_{10}) was calculated. Values of 1.87 ± 0.09 for SC1 and 1.55 ± 0.02 imply that heat applied before neon radiation is more effective in reducing cell survival than when cells were irradiated by x-rays alone. Parallel experiments using the temperature-sensitive mutant, tsH1 (Figure 12A), indicated that in the absence of protein synthesis, there was no modification of the shoulder of the survival curve. The ratio of final slopes D_o (TER) was 1.35 ± 0.06 which was comparable to that of SC1 cells. The values for TEF_{10} and RBE_{10} were both reduced compared to SC1 cells (Table 3).

When the sequence of radiation and heat was reversed such that heat was applied immediately after exposure of the cells to the 32 keV/μm neon radiation (Figure 12B), the TEF_{10}, TER and RBE_{10} values for SC1 cells were significantly greater than those obtained when heat was applied prior to neon irradiation (Figure 12A), or heat applied prior to x-ray exposure (Figure 11). A comparison of thermal enhancements and biological effectiveness for the two

different sequences is illustrated for both cells in Table 3. There were no significant differences in TEF_{10} and RBE_{10} of the tsH1 cells regardless of the sequence of application of heat and 32 keV/μm neon radiation.

Table 3 Comparison of TEF_{10}, TER and RBE_{10} as a function of sequence of application of heat and 32 keV/μm neon radiation.

	41.5°C **before** neon radiation			41.5°C **after** neon radiation		
	TEF_{10}	TER	RBE_{10}	TEF_{10}	TER	RBE_{10}
CHO-SC1	1.50 ± 0.08	1.26 ± 0.5	1.87 ± 0.09	1.98 ± 0.08	1.72 ± 0.11	2.47 ± 0.10
CHO-tsH1	1.24 ± 0.03	1.35 ± 0.06	1.55 ± 0.02	1.27 ± 0.03	1.29 ± 0.09	1.59 ± 0.03

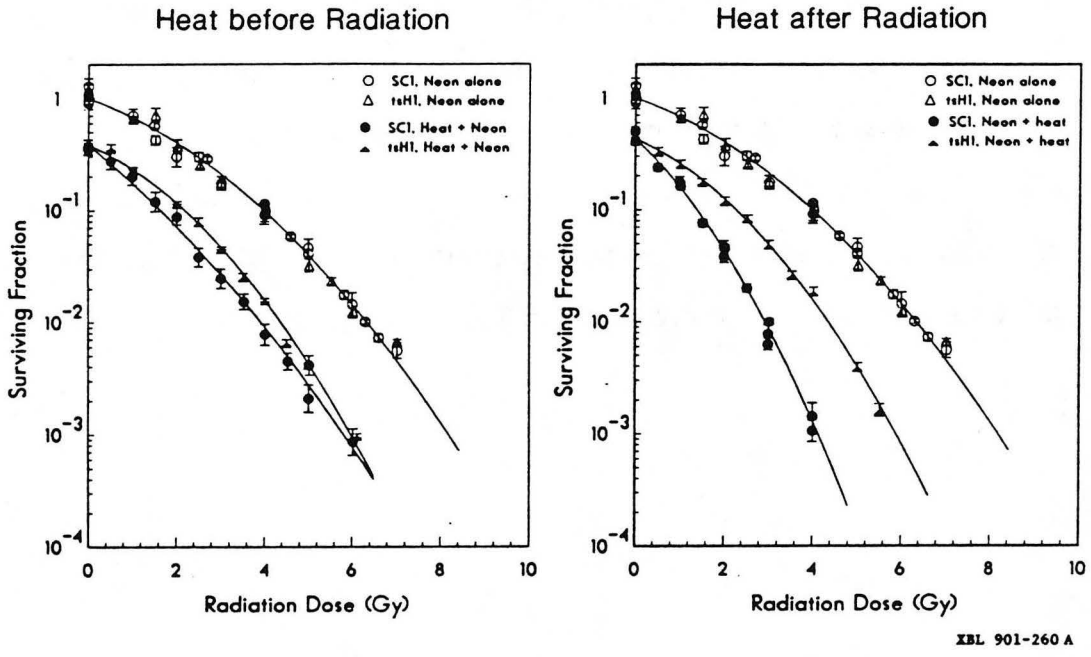

Figure 12 Survival response of CHO-SC1 (○) and CHO-tsH1 (△) cells after 425 MeV/μm neon ions at plateau position of the Bragg curve with an LET of 32 keV/μm. Solid symbols for both cells represent survival data for heat and radiation. Left panel: Survival curves of both cells heat treated (4 hrs at 41.5°C) before neon irradiation; Right panel: Survival curves of both cells irradiated with neon ions followed by heat treatment (4 hrs at 41.5°C). The origins of the heated curves are plotted to reflect the amount of heat toxicity when cells are subjected to heating alone.

In a separate series of experiments, cells were irradiated with neon ion beams with LETs ranging from 32 keV/μm to 183 keV/μm followed immediately by heat treatment of 4 hrs at 41.5°C. Figure 13 shows four panels of survival curves obtained for LET = 32 keV/μm, 80 keV/μm, 100 keV/μm and 183 keV/μm. Each experiment was repeated at least twice. For all the four LETs examined, the SC1 and tsH1 survival response to neon radiation alone is the same, ie. both cells show the same radiosensitivity to neon alone. The LQ and SHMT parameters obtained from the fitted curves of neon radiation alone, and neon radiation combined with 41.5°C for the SC1 and tsH1 survival curves are presented in Table 4 and Table 5.

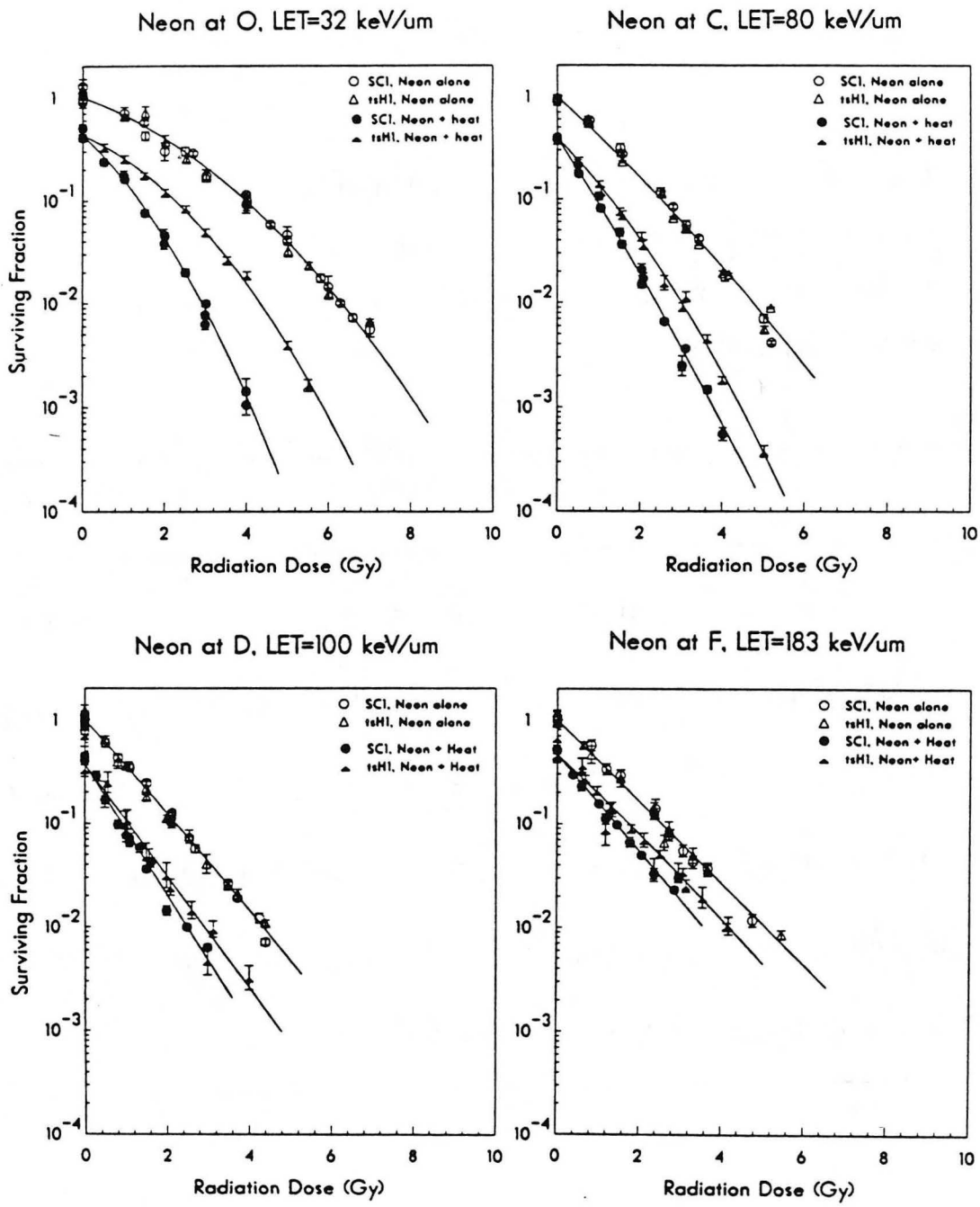

Figure 13 Survival curves showing the variations of cellular survival radiation response with or without heat treatment with increasing LET from 32 keV/μm up to 183 keV/μm.

Table 4 α and β values generated from LQ model, and extrapolation number, n, initial slope D_q and final slope D_o values generated from SHMT model to the survival data from CHO-SC1 cells treated with radiation and 4 hrs of heat at 41.5°C

LET	α (10^{-3})	β (10^{-6})	n	D_q (Gy)	D_o (Gy)
x-rays + heat	0.50 ± 0.22	0.10 ± 0.05	2.81 ± 2.1	0.88 ± 0.53	0.85 ± 0.12
30 keV/μm + heat	0.84 ± 0.27	0.15 ± 0.08	2.06 ± 1.3	0.47 ± 0.35	0.66 ± 0.1
80 keV/μm + heat	1.46 ± 0.25	0.03 ± 0.07	1.2 ± 0.45	0.11 ± 0.22	0.62 ± 0.04
100 keV/μm + heat	1.45 ± 0.47	0	1.0 ± 0.66	0 ± 0.46	0.69 ± 0.12
183 keV/μm + heat	1.07 ± 0.25	0	1.0 ± 0.39	0 ± 0.36	0.93 ± 0.13
heat + 30 keV/μm	0.733 ± 0.2	0.05 ± 0.04	2.13 ± 0.81	0.67 ± 0.3	0.89 ± 0.06

Table 5 α and β values generated from LQ model, and extrapolation number,n, initial slope D_q and final slope D_o values generated from SHMT model to the survival data from CHO-tsH1 cells treated with radiation and 4 hrs of heat at 41.5°C

LET	α (10^{-3})	β (10^{-6})	n	D_q (Gy)	D_o (Gy)
x-rays + heat	0.23 ± 0.25	0.08 ± 0.04	5.2 ± 6	1.76 ± 0.9	1.07 ± 0.23
30 keV/μm + heat	0.40 ± 0.10	0.11 ± 0.02	3.5 ± 2.5	1.09 ± 0.5	0.88 ± 0.13
80 keV/μm + heat	0.96 ± 0.14	0.09 ± 0.03	1.74 ± 0.66	0.39 ± 0.24	0.70 ± 0.05
100 keV/μm + heat	1.24 ± 0.5	0	1.0 ± 0.81	0	0.81 ± 0.16
183 keV/μm + heat	0.85 ± 0.17	0.02 ± 0.04	1.15 ± 0.37	0.15 ± 0.34	1.06 ± 0.09
heat + 30 keV/μm	0.39 ± 0.07	0.10 ± 0.01	4.53 ± 2.2	1.26 ± 0.31	0.84 ± 0.08

An LET-dependent synergistic effect occurred on cell survival in cultures exposed to neon radiation followed by heating. For the range of LETs studied, heated SC1 cells consistently show greater radiosensitivity compared to that of the heated tsH1 cells. Table 6 compares the TEF and TER of heated CHO-SC1 and CHO-tsH1 cells with neon irradiation using neon radiation alone as the standard. Figure 14 illustrates the variation of thermal enhancements with LET from x-rays to 183 keV/μm. The TEF of SC1 at LET = 32 keV/μm was significantly greater than that of x-rays. As the LET increased above 32 keV/μm, the TEF decreased down to about 1.0 at 183 keV/μm. This LET-dependence of TEF was not clearly demonstrated in the tsH1 cells.

Table 6 Comparison of TEF, and TER of CHO-SC1 and CHO-tsH1 cells irradiated with 425 MeV/u Neon ions with different LETs, followed by heat treatment of 4 hrs at 41.5°C.

LET (keV/μm)	CHO-SC1		CHO-tsH1	
	TEF	TER	TEF	TER
x-rays	1.71 ± 0.08	1.38 ± 0.09	1.19 ± 0.06	1.11 ± 0.11
32	1.98 ± 0.08	1.72 ± 0.11	1.27 ± 0.03	1.29 ± 0.09
80	1.64 ± 0.09	1.56 ± 0.06	1.24 ± 0.05	1.38 ± 0.05
100	1.40 ± 0.11	1.35 ± 0.11	1.20 ± 0.12	1.15 ± 0.11
183	1.19 ± 0.06	1.11 ± 0.07	0.99 ± 0.05	0.97 ± 0.05

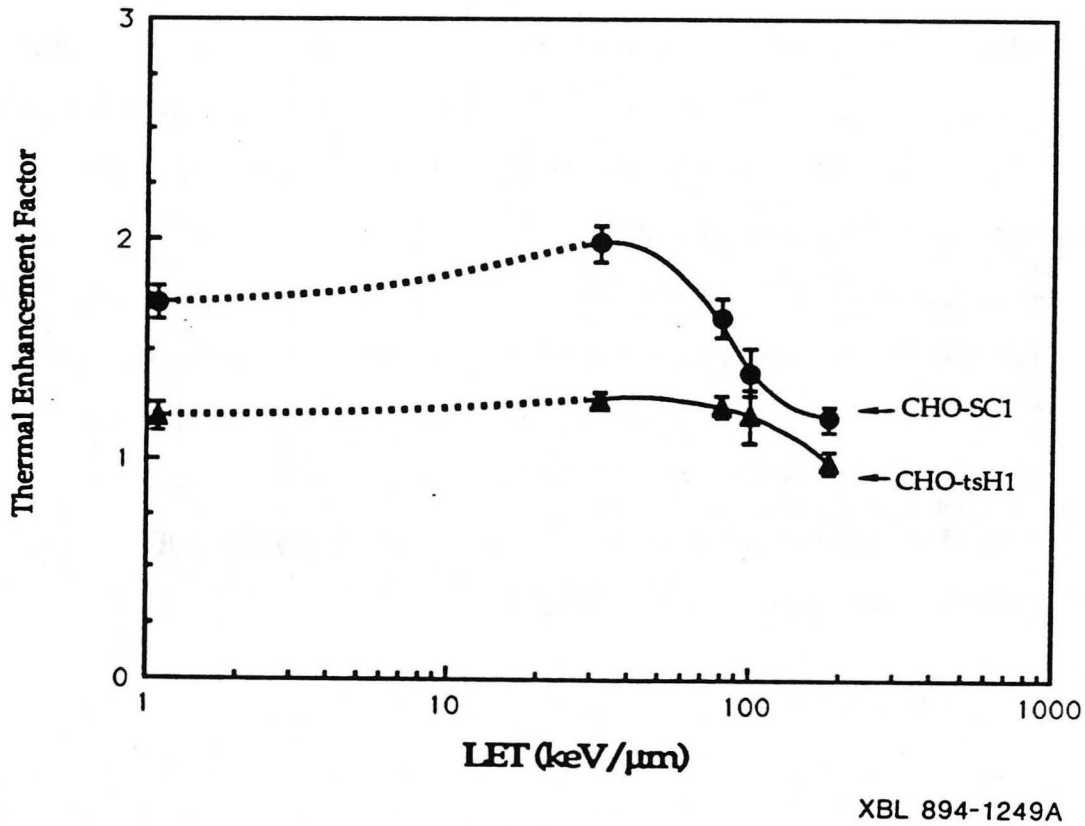

Figure 14 The variation of thermal enhancement with LETs for CHO-SC1 cells and CHO-tsH1 cells. The thermal enhancement factors were calculated using the radiation dose to give 10% survival for the heated survival curves compared to that of the unheated survival curve. Control x-ray TEF values are plotted at 1 - 2 keV/μm.

Using the survival curve for exposure to x-rays alone as the reference survival curve, the relative biological effectiveness at 10% survival (RBE_{10}) was calculated for each cell line irradiated with neon ions alone. Tables 8 and 9 list the RBE_{10}s calculated at 10% survival and the RBE calculated from the ratio of final slopes when the heavy ion survival curves were compared to those of x-rays. The RBE_{10} vs LET curve is in agreement with published reports for CHO cells (Blakely et al., 1984), with the peak RBE_{10} occurring between 100 keV/μm and 200 keV/μm (Figure 15). CHO-SC1 cells, irradiated and post-treated with heat, showed a significantly higher peak RBE_{10} of 3.25 ± 0.15. Furthermore, this maximum response occurred at a lower LET of 80 keV/μm. In the absence of protein synthesis in the tsH1 cells, the peak RBE_{10} was slightly higher ($RBE_{10}= 2.67 \pm 0.25$) than for the unheated cells, and this maximum RBE_{10} appears to occur at a similar LET to that of the unheated controls (100 - 200 keV/μm).

Table 7 Relative Biological Effectiveness (RBE_{10}) at 10% survival

LET (keV/μm)	CHO-SC1/CHO-tsH1	heated CHO-SC1	heated CHO-tsH1
x-rays	1.0 *	1.71±0.08	1.19±0.06
30	1.25±0.02	2.47±0.10	1.59±0.03
80	1.98±0.06	3.26±0.15	2.45±0.07
100	2.23±0.05	3.12±0.23	2.67±0.25
183	1.95±0.06	2.32±0.9	1.92±0.08

* 150 kVp x-rays used as reference

Table 8 Relative Biological Effectiveness using D_0 ratios

LET (keV/μm)	CHO-SC1/CHO-tsH1	heated CHO-SC1	heated CHO-tsH1
x-rays	1.0*	1.38 ± 0.09	1.11 ± 0.11
32	1.05 ± 0.029	1.80 ± 0.11	1.35 ± 0.09
80	1.22 ± 0.02	1.90 ± 0.07	1.69 ± 0.06
100	1.27 ± 0.03	1.71 ± 0.14	1.46 ± 0.13
183	1.15 ± 0.03	1.27 ± 0.08	1.11 ± 0.05

* 150 kVp x-rays used as reference

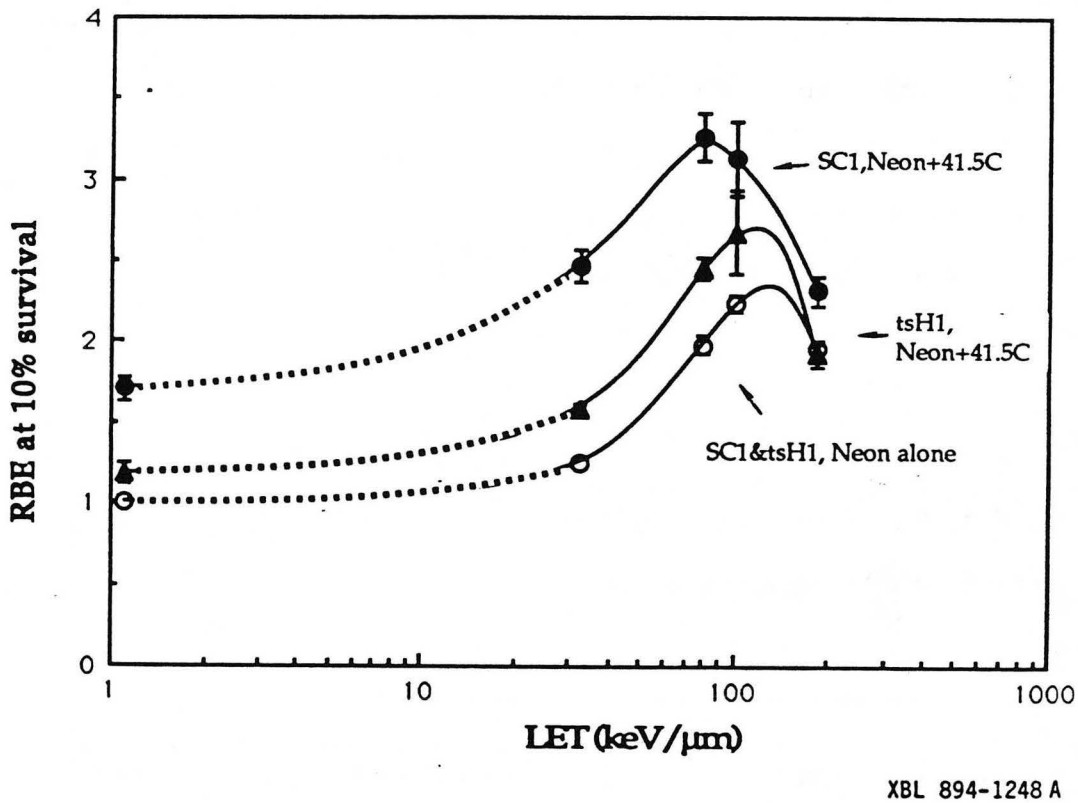

Figure 15 The variation of RBE_{10} values with LETs for CHO-SC1 cells and CHO-tsH1 cells. Empty symbols show the RBE_{10} of both cells after radiation alone. Solid symbols show the RBE_{10} variations of CHO-SC1 (●) and CHO-tsH1 (▲) cells that were irradiated and followed by hyperthermic treatments. x-ray control RBE_{10} values are plotted at 1 - 2 keV/μm.

Inhibition of protein synthesis using cycloheximide

Protein synthesis was shut down in CHO-SC1 cells by pretreating with cycloheximide, followed by neon irradiation and the same heat treatment in the presence of cycloheximide. SC1 cells exposed to cycloheximide and heated for 4 hrs at 41.5°C showed no heat toxicity and in fact, may demonstrate slightly enhanced survival. A comparison of the RBE_{10} of cycloheximide- and heat-treated SC1 cells and that of heat-treated tsH1 over the LET range studied is shown in Table 9. There were no significant differences in RBE_{10} values between the two systems.

Table 9 Comparison of RBE_{10} of CHO-SC1 treated with cycloheximide and CHO-tsH1. Both cells were irradiated with 425 MeV/u neon followed immediately by heating at 41.5°C.

LET (keV/μm)	SC1 + cycloheximide	tsH1
32	1.67 ± 0.06	1.59 ± 0.03
80	2.73 ± 0.17	2.45 ± 0.07
100	2.89 ± 0.12	2.67 ± 0.25
180	1.99 ± 0.2	1.92 ± 0.08

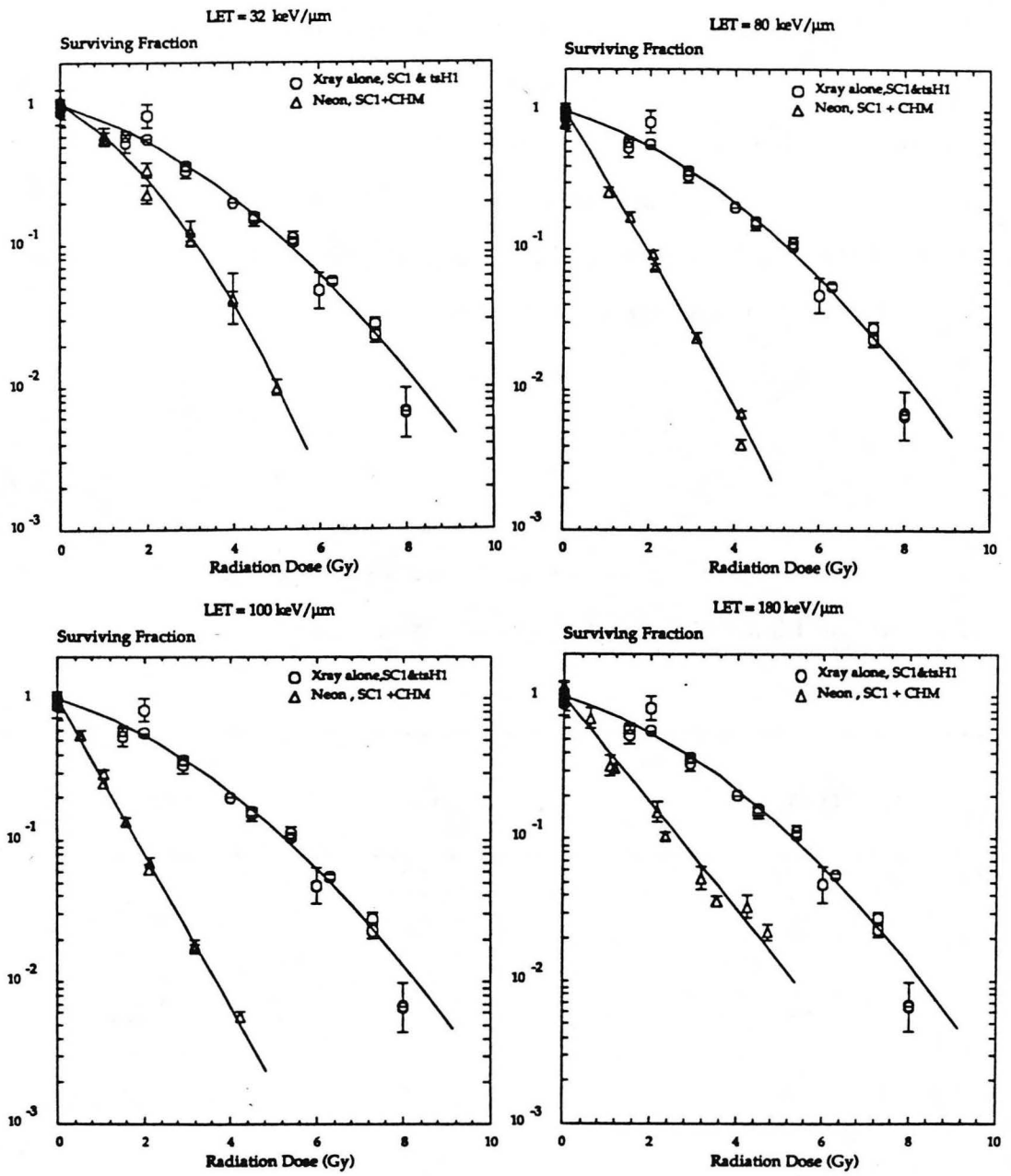

Figure 16 CHO-SC1 cells treated with cycloheximide 2 hrs prior to irradiation to inhibit protein synthesis. These cells were irradiated at four different LETs and further heat treated for 4 hrs of heat treatment at 41.5°C.

High-LET hyperthermic radiosensitization at 42°C

We also compared the effectiveness of two different heat treatments: 4 hrs at 41.5°C vs. 1.5 hrs at 42°C in the wild type SC1 cells. These two heat treatment schedules are known to be equitoxic as seen in the thermal response curves (Figures 1 & 2). The RBE obtained for the LETs (Table 10) studied showed that indeed, the synergistic effects of combined treatment is equivalent under both heating conditions .

Table 10 Comparison of RBE_{10} of CHO-SC1 irradiated with neon ions followed by 1.5 hrs at 42°C vs. 4 hrs at 41.5°C

LET (keV/μm)	1.5 hrs at 42°C	4 hrs at 41.5°C
32	2.61 ± 0.10	2.47 ± 0.1
80	3.06 ± 0.24	3.26 ± 0.15
100	3.20 ± 0.26	3.12 ± 0.23
180	2.20 ± 0.32	2.32 ± 0.09

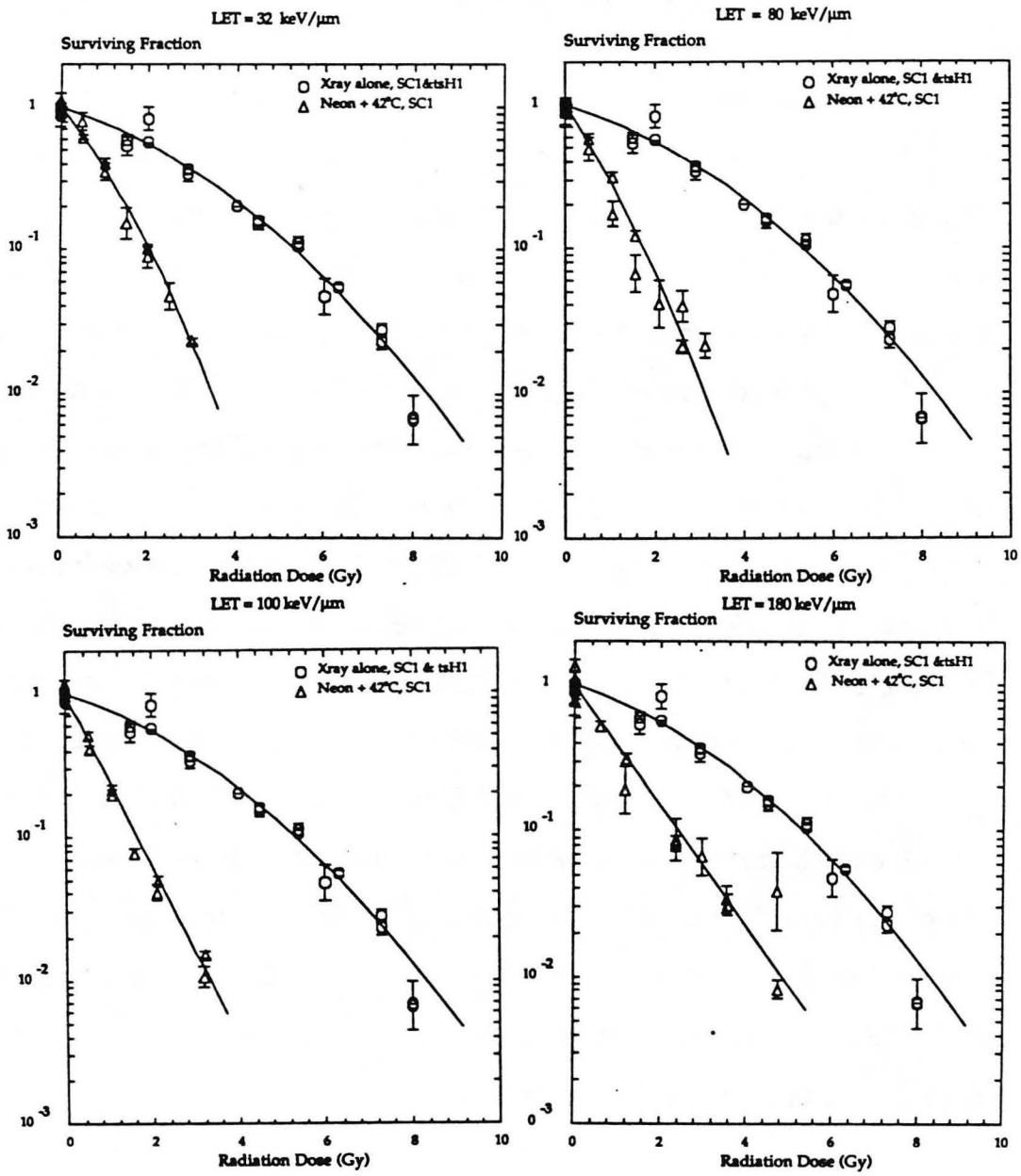

Figure 17 CHO-SC1 cells irradiated with four different LETs and post-treated with 1.5 hrs at 42°C.

DISCUSSION

Thermal response of CHO-SC1 and CHO-tsH1 cells

These experiments show that thermotolerance can be triggered in CHO-SC1 cells under 3 conditions: 1) a chronic exposure to 41.5°C, 42°C, 42.5°C (Figure 1, panel A); 2) an acute triggering heat shock of 15 min at 45°C, followed by a development time at the permissive temperature of 35°C and a challenge dose of 25 min at 45°C (Figure 3, panel A); and 3) acute triggering heat shock of 5 min at 45°C, followed by time of incubation at the non-permissive temperature of 40°C, and challenged by a 25 min exposure to 45°C (Figure 5, panel A). The resultant survival responses are in agreement with the earlier results of Henle et al. (1978) in that the kinetics of induction of thermoprotection are highly dependent on the conditions of heat treatment including the triggering temperatures, and the duration and times of incubation. It is known that the immediate response to heat shock is the inhibition of rRNA synthesis, the reduction of translation of pre-existing mRNA and the reprogramming of transcription for the preferential synthesis of new heat shock mRNAs (McKenzie et al., 1975; Petersen and Mitchell, 1981; Nover 1984,). This response can be seen in the SC1 cells; however, for the tsH1 cells, this response is absent, since there is no new protein synthesis, and therefore no heat shock protein synthesis.

Survival responses of the mutant tsH1 cells after exposure to heating at 41.5°C, 42°C, and 42.5°C, show exponential killing after an initial shoulder with continuous heating. They show no development of the biphasic response which is an indication of the lack of thermotolerance (Figure 1, panel B). The broader shoulders and decreased terminal slopes of all three

survival curves of tsH1 cells compared to that of SC1 cells indicated that these mutants were inherently more resistant to heat killing than the wild-type cells. The studies of the rate of ^{3}H-leucine uptake at 42°C indicate that protein synthetic activity was depressed. PAGE gels of 42°C-treated tsH1 cells also showed that there was no significant protein synthesis 2 to 4 hours after the hyperthermic treatments. This suggests that there was no overexpression of any proteins under these hyperthermic conditions. The total protein measurements of control cells show that both cell lines under normal conditions have very similar total protein content. A comparison of control lanes of PAGE gels for SC1 and tsH1 cells indicate an increased uptake of ^{35}S-met for protein synthesis in the tsH1 cells over that of SC1 under untreated control conditions. Pool sizes of the various amino acids for either cell line have not been measured. Therefore, it cannot be concluded at this time whether this difference in protein synthetic activity could account for the increased resistance in the thermal response in the mutant cells compared with that of the wild-type cells under the same conditions.

When tsH1 cells were exposed to an acute trigger temperature dose of 45°C for 5 min, followed by an incremental incubation period of 0 to 7 hrs. at the permissive temperature of 35°C, and then challenged with an additional heat dose of 45°C for 27 min, thermotolerance was expressed that was equal in magnitude to that seen in the wild-type SC1 cells (Figure 4, panel B). Vidair and Dewey (1989) reported similar findings using the same cell system. In separate experiments, mutant cells were exposed to the same acute heat exposures of 45°C separated by an incubation period at the non-permissive temperature of 40°C for 0 to 7 hours (Figure 6, panel B), tolerance was also developed, but to a much lesser extent compared with SC1 cells under the

same conditions. The decay of this reduced thermotolerance is also more rapid compared to the SC1 cells. Therefore, the presence of active protein synthesis is not required for the initial onset of thermotolerance by acute heating, but is needed for the sustenance of thermotolerance for prolonged hyperthermia. At 40°C - 42°C, the % rate of protein synthesis in tsH1 cells was depleted to less than 10% of the initial control (Figure 7)(Yezzi, et al., 1988), and no hsps were detected (Figure 6). Anderson and Baranowski (1984) demonstrated that acidic nuclear proteins are still being synthesized at 40°C up to 1 hr of heating. These proteins may account for a portion of the residual activity in ^{3}H-leu uptake and it has been suggested that they may include some translationally-regulated "prompt" hsps associated with the nuclear matrix-intermediate filaments, distinct from the transcriptionally-regulated hsps (Reiter and Penman, 1983). Such proteins may also contribute to the transient resistance to subsequent heat shock seen in these mutants.

In the absence of synthesis of new cytosol proteins, the translocation of constitutive proteins present in the cell may play a role in the transient thermoresistance observed in the mutant cells. There have been reports of an increase in nuclear protein content with heat stress and the development of thermotolerance (Reiter and Penman, 1983; Murane and Li, 1985). Suggestions of translocation of proteins to stabilize cell membranes have been made by Yatvin et al. (1987) in *E. coli* and the same mechanism could also contribute to thermal resistance in the tsH1 cells. However, in the absence of new cytoplasmic protein synthesis, the existing pool of constitutive proteins that are responsible for the transient thermotolerance can be depleted, leading to the decay of tolerance. Enhanced protein glycosylation after exposure to heat, as proposed by Henle et al. (1988), could also be the mechanism leading

to the transitory tolerance observed in the tsH1 cells in the absence of new cytosol protein synthesis.

To date, the only known stable mammalian mutants that do not develop thermotolerance are those of CHO selected by Harvey and Bedford (1988) and ubiquitin-deficient mutant cells ts85, derived from mouse mammary carcinoma (Mizuno et al., 1989). The mutant cells selected by Harvey and Bedford (1988) are morphologically different from their parental CHO cell line, and cytoskeletal alterations are thought to contribute to their altered difference in thermoresponse. For the ubiquitin-deficient temperature-sensitive mutant ts85, the lack of ubiquitin at the non-permissive temperature of incubation is thought to contribute to the lack of thermoprotection. tsH1 cells are morphologically similar to the SC1 parent and have approximately the same cell population doubling times. The only known difference between these CHO-SC1 and CHO-tsH1 cells is the specific defect in the protein synthetic machinery in the tsH1 cells. Our experimental results indicate that these cells can be used to demonstrate that the synthesis of new cytosol proteins is not required for the initial onset of thermotolerance, but is necessary for the sustenance of tolerance. Constitutive levels of proteins and/or the low residual level of prompt nuclear protein synthesis appear to be adequate for the initial development of thermotolerance, but these protein pools may be quickly depleted by heat stress. These tsH1 mutant cells, with SC1 as the control, can be useful in further investigating the relative involvement of protein synthesis in the nucleus and cytosol of mammalian cells without the perturbation of drugs.

High-LET radiation and hyperthermia

The combined effects of high-LET radiation and hyperthermia are not well understood and no synergistic effects have previously been reported (Hahn, 1982). An important finding of these investigations is that the interaction between ionizing radiation and high temperature is radically different when high temperature is applied after irradiation rather than before irradiation. Hyperthermia at 41.5°C administered prior to high-LET neon radiation (32 keV/μm) has a smaller effect on cellular survival *in vitro* following radiation alone than is observed for combined exposure of heat and low-LET radiation (Table 5 and 6). This is in agreement with the results of Gerner and Leith (1977). However, when heat was applied immediately after 32 keV/μm neon irradiation, synergism was observed between the two modalities. According to International Commission on Radiation Units and Measurements (ICRU) report #30 on the Quantitative Concepts and Dosimetry in Radiobiology, the definition of synergistic action is a dose-effect dependence in which the additional effect produced by neon radiation, in combination with a fixed dose of heat at 41.5°C, is more effective than neon radiation alone when account is taken of a dose of neon biologically equivalent to that of heat. Both the thermal and relative biological enhancements at the 10% survival level are greater in the neon combined with heat treatments compared to that of neon alone, or x-rays alone. When comparing RBE_{10} and TEF values of SC1 and tsH1 cells exposed to heat before or after radiation, it was found that in the absence of protein synthesis in the tsH1 mutant, no difference in either the TEF or the RBE_{10} between the two sequences was observed. The sequence of administering low-LET radiation and heat has been recognized as important (Dewey et al., 1977). Low heat

(40°C-43°C) is more effective when given post irradiation, due to an acceleration of damage fixation; and high heat (above 43°C) is more effective when administered before radiation, resulting in the inactivation of repair processes. The sequence of application of heat and radiation is shown in these experimental results to be also important for high-LET radiation studies.

Recent evidence suggests that the genetic regulatory domains associated with recovery from heat damage and those associated with repair of ionizing radiation damage may overlap. McClanahan and McEntee (1986) showed that two genes in yeast of unknown function were both transcriptionally regulated by DNA damage and heat shock. However, not all heat-inducible genes, e.g., YG100 and YG102, are inducible by 4NQO (4-nitroquinoline-1-oxide, a DNA damaging agent) and not all DNA damage-inducible genes e.g., DIN1, are inducible by heat shock (McClanahan and McEntee, 1986). Yeast, exposed to low temperatures, can also be induced by radiation to exhibit thermoresistance, and can also be induced by heat to exhibit radioresistance. Both the induction of resistance and the decay of response to both radiation and heat require protein synthesis (Mitchel and Morrison, 1982). Since DNA damage as diverse as that produced by ionizing radiation or UV both induce thermotolerance, heat-induced alterations in DNA or in DNA-dependent chromosomal organization may be the signal for heat induction of thermotolerance in yeast and other eukaryotes (Mitchell and Morrison, 1984) These authors also suggest recognition and repair of DNA by recombinational repair is not part of a signal which initiates an increase in resistance to heat. To date, such coinductions of heat and radiation responses have not been reported in mammalian cells due to the

complexity of the cell systems involved. However, these reported observations in yeast suggest that the control for heat damage recovery, and radiation repair, though separate for the most part, may contain a subset of overlapping coinduction regions.

Possible mechanisms of action

The experimental results presented here provide evidence that protein synthesis is involved in the hyperthermic radiosensitization with x-rays, and also with each of the various high-LET radiations studied, as shown by the diminished sensitization for cell killing in the absence of active protein synthesis in the CHO-tsH1 mutant cells or cycloheximide-treated CHO-SC1 cells, compared to that of the untreated CHO-SC1 control cells. In exposing tsH1 cells to non-permissive temperatures of heat incubation, total protein synthesis is inhibited; therefore, it is not possible to evaluate the relative involvement of specific proteins, e.g. heat shock proteins (hsps), or x-ray-induced proteins (XIPs). As is inherent in all cell survival studies where cell killing is used as an endpoint for the assay, the processes leading to ultimate cell death are unknown. Similarly, the results obtained in these investigations do not allow one to distinguish whether the presence of active protein synthesis increases the probability of misrepair or damage fixation, hinders correct repair of the damaged genome, or plays a role in the expression of the phenotype of the damaged genome which might otherwise be silent in the absence of heat.

Some speculation on the possible mechanisms of action underlying the observations made here would likely involve the functional role of the

proteins that are induced in the presence of heat. When cells are exposed to heat alone, there is an increased translocation of proteins, especially specific hsps, into the nucleus of the cells (Velazquez and Lindquist, 1984; Lewis and Pelham, 1985). Some of the functions of hsps, especially hsps 70s and 90s, have been reported to be associated with the transport and assembly of newly synthesized proteins within the cells (Flynn et al., 1989; Beckmann et al., 1990). Furthermore, the functional activity of hsp70 has been shown to be important for cells to survive thermal stress (Riabowol et al., 1988). Initiation of replication and nuclear processing are inhibited after hyperthermia (Warters 1988), and may be associated with the increase in binding of proteins to DNA as evidenced by the decreased extractability of proteins (Wong and Dewey, 1982; Chu et al., 1990). The increased association of histone and non-histone chromosomal proteins with nuclear matrix intermediate filaments and the genome may cause alterations in the structural organization of chromosomes, thereby causing difficulties in the recognition and accessibility of catalytically-active repair enzymes to the sites of radiation-induced DNA damage.

In the absence of active protein synthesis, it is possible that the translocation of native cytosol proteins to the nucleus is limited, resulting in a decreased inhibition in proficient repair of DNA damage. The level of protection from thermal sensitization after neon irradiation was comparable whether protein synthesis was inhibited by the addition of cycloheximide or by temperature change in the tsH1 cells. Cycloheximide alone protects cells from heat damage resulting in a lack of heat toxicity and a small increase in initial survival without radiation. Similar results have been reported by Chu and Dewey (1987) and Lee et al. (1987). Such heat protection has been

suggested to be related to differences in levels of induced cellular hsps after cells are released from hyperthermia (Lee et al., 1987). In the temperature-sensitive mutant, active protein synthesis is shut down when the temperature of incubation is elevated to ≥ 40°C , and a thermal dose of 4 hrs at 41.5°C alone is equitoxic to both the mutant and its wild-type parent. The pattern of inhibition and recovery of the rate of total protein synthesis with heating is similar in both cells, but the question as to whether there is differential synthesis of specific proteins after exposure to high-LET irradiation followed by heat is still under investigation.

The differential sensitivity to radiation-induced lethality of cells in different phases of the cell cycle may be an important aspect in the cytotoxic effect of combined exposure to low-LET radiation and heat. The most radioresistant cells are those in S-phase, and S-phase cells are the most heat-sensitive. For high-LET radiation exposure, the cell cycle fluctuation in radiation sensitivity is LET-dependent and is reduced at 183 keV/μm (Blakely et al., 1984; 1985; 1989). High-LET radiation has been shown to cause a dose- and LET-dependent G_2-arrest in exponentially growing cells (Lucke-Huhle et al., 1979). The maximum effective RBE for this G_2+M block for CH-V79 cells was shown to occur at 100 keV/μm. Roots et al. (1979) have reported that the production of nonrejoining strand breaks increased with increasing LET with the maximum effect at about 100 keV/μm. Recovery from sublethal (SLD) and potentially lethal damage (PLD) in synchronized human T-1 cells is LET-dependent (Blakely et al., 1985; 1989). All these results suggest that the repair of high-LET radiation-induced DNA damage is specific to discrete stages of the cell cycle, and is unlike the repair of x- or γ- radiation-induced damage which can be repaired during all phases of the cell cycle (Lett and Sun, 1970).

Hyperthermia alone has been shown to cause perturbations in the cell cycle, e.g., the inhibition of DNA synthesis in synchronized CHO cells (Wong and Dewey, 1982; Wong et al., 1989,). The effects of high-LET irradiation on an exponentially growing cell culture are thus expected to cause an LET-dependent redistribution of cells to the thermosensitive S/G_2 phase. The differences in sensitivities between the wild-type cells and the temperature-sensitive mutant cells suggest that active protein synthesis may be involved in this partial synchronization of cells after heavy-ion radiation and hyperthermia.

Possible clinical application in cancer therapy

One of the major limitations in administering hyperthermia and/or sparsely ionizing radiation e.g., x-rays, effectively to interstitial tumors in the clinic is the problem of heterogeneous temperature and dose distributions at internal tumor sites, compounded by an excessive skin dose. With careful treatment planning, the dose of heavy ion radiation delivered to a localized tumor site can be controlled, with minimum dose delivered to the surrounding tissue. Therefore studies of the effectiveness of combining hyperthermia with heavy-ion radiation could have clinical significance.

Recent advances in the use of hyperthermia in the clinic for cancer therapy have drawn attention to the difficulties involved in administering a localized heat dose to interstitial tumors due to the characteristic exponential decrease in heat dose with increasing distance of penetration in tissue. A variety of different physical heating methods are now being tested with some success to compensate for the loss of thermal dose at various depths of

penetration. These include the use of ultrasound, magnetic induction, high frequency current fields, microwaves, and implantation of thermally regulated ferromagnets (Lilly et al., 1985) and a variety of combined configurations for exposure e.g., focussing, and the use of multi-applicators. Other means of administering heat at depth involve the invasive surgical procedure of exposing the tissue to be treated for the positioning of thermoprobes.

The experimental results obtained in this thesis suggest that low heat at 41.5°C or 42°C, applied after high-LET irradiation is more effective in cell killing that heat applied alone, heat applied after x-ray irradiation and heat applied before high-LET irradiation. The ratio of maximum RBE_{10} for cell survival to the RBE_{10} for lower LET radiation e.g. Bragg plateau, under the same treatment conditions, gives a relative gain factor when considering the merit of using high-LET radiations in cancer therapy. For heavy-ion radiation alone and using x-rays as standard, the RBE_{10} is about 2.2 at 100 keV/μm. The ratio of maximum RBE_{10} for neon irradiated and heat treated SC1 cells compared to the RBE_{10} of x-irradiated and heat treated SC1 cells is about 2.0 at 80 keV/μm. For the tsH1 cells, this ratio is about 1.9 at 100 keV/μm. These values suggest that the combination of heat and high-LET radiation is at least as effective as high-LET radiation alone, with a higher overall RBE_{10}. Such LET-dependence of enhanced thermal sensitization and RBE could possibly be useful in a combined high-LET radiation and hyperthermia treatment strategy of some radioresistant cancers.

The sequence of application of the high-LET radiation and hyperthermia is important. Similar to low-LET cancer radiotherapy, it is recommended that low temperature hyperthermia be administered after the

high-LET radiation exposure in order to maximize the effectiveness of the combined treatment, and to minimize the changes in tumor physiology that will lead to an increase in the complexity of the radiation response.

CONCLUSION

In conclusion, the central findings of this thesis include:

1. The presence of active protein synthesis is not required for the initiation of thermotolerance in a Chinese hamster cell system *in vitro,* but is necessary for the persistence of this transient resistance to heat.

2. The absence of the biphasic survival response in chronic hyperthermic exposure does not necessarily imply that there is no thermotolerance.

3. The sequence of application of low heat at 41.5°C and high-LET radiation is important when considering synergistic effects in cell killing. Heat applied after neon irradiation at an LET of 32 keV/μm is more effective than heat applied before neon irradiation.

4. With cell killing as an end-point, both the thermal enhancement and the relative biological effectiveness for the combined heat and high-LET radiation treatments are LET-dependent. Maximal thermal sensitization appears to occur at about 80 keV/μm, while the RBE for heat and high-LET radiation effects peaks at about 100 keV/μm.

5. The presence of active protein synthesis is important for the enhanced thermal and radiation effects. In the absence of protein synthesis, the magnitude of RBE and TEF is reduced.

6. Protein synthesis inhibition, whether by activating the defect in the leucyl-tRNA synthetase Chinese hamster cell system, or by cycloheximide blocking, appear to lead to similar cell survival responses when cells are subjected to heat and neon irradiation.

7. Equitoxic heat doses of 4 hrs at 41.5°C or 1.5 hrs at 42°C, when combined with neon irradiation, result in the same thermal sensitization and biological effectiveness.

8. High-LET-induced cellular damage is modifiable by hyperthemia and protein synthesis plays an important role in modulating this response.

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APPENDIX A

2X Sample Buffer

To make 10 mls for 0.5 ml aliquots/sterile eppendorf tube (1.5 ml capacity).

Store frozen at -20°C

	Volume	Final Concentration.
SDS (10% stock solution)	3 ml	3%
Stacking gel buffer (0.5M Tris buffer, pH6.8)	1 mls	50 mM
Glycerol	5 mls	50% v/v
β mercaptoethanol	0.2 mls	2%
Bromophenol Blue	0.02 gm	0.2%
Glass distilled water (DGW)	0.9 mls	

Add equal amount directly to sample before loading to gels

Lysis Buffer

To make 100 mls

	Volume	Final Concentration.
10% SDS	10 mls	1%
0.5M Tris buffer, pH 6.8	10 mls	50 mM
1M EDTA	0.2 mls	2 mM
Glass distilled water (DGW)	79.8 mls	

Polyacrylamide Gel Electrophoresis Solutions

1M Tris-Base, pH 8.9

To make 500 mls (M. W. =121.1)

1. Dissolve 60.55 gm of Tris-base in 250 ml of DGW,
2. Adjust pH to 8.9 with 6N HCl,
3. Bring volume up to 500 mls.

1M Tris-Base, pH 6.7

To make 500 mls

1. Dissolve 60.55 mls of Tris-base in 250 mls DGW,
2. Add approximately 50 mls of 6N HCl and pH to 6.7,
3. Bring volume up to 500 mls.

30% Acrylamide:

To make 250 mls:

1. Dissolve 75 gms of acrylamide, (CAUTION: potent neurotoxin), in distilled water to make volume up to 250 ml,
2. Add 6.25 gms of Amberlite* (MB-3) to solution and stir for 30 min to remove impurities,

   Filter with 0.45 Nalgene or Whatman filter.

*Amberlite MB-3: is a mixture of Amberlite IR-120, a cation exchange resin (H^+-form) and Amberlite IRA-410, an anion exchange resin (Hydrogen form). This mixture also contains thymolphthalein indicator which changes color from deep green to yellow as the deionizing capacity of the resin is exhausted.

1% Bis-Acrylamide

To make 50 mls: Dissolve 0.5 gms Bis-acrylamide in distilled water to make up to 50 mls.

10% SDS

To make 500 ml (M.W.= 288.38)

Dissolve 50 gms SDS (BioRad #161-0301, or Sigma M2697), in distilled water to make up to 500 mls. Filter sterilize.

10% Ammonium Persulfate

To make 50 ml: Dissolve 5 gm of ammonium persulfate in distilled water and make up to 50 ml of solution

Running buffer

To make 10 liters:

1. Combine 30.33 gm Trizma base, 142.66 gm glycine, 10 gm SDS,
2. Adjust to pH to 8.6
3. Make up to 10 liters with glass distilled water.

For 12.5% PAGE gels:

Stacking gel

	1 gel	2 gels
30% acrylamide	1.5 ml	3.0 ml
1% bis-acrylamide	0.6 ml	1.2 ml
1M Tris pH 6.7	1.5 ml	3.0 ml
Glass distilled water	8.75 ml	17.5 ml
10% SDS	125 µl	250 µl
ammomium persulphate	62.5 µl	125 µl
Temed	20 µl	40 µl

Resolving gel

	1 gel	2 gels
30% acrylamide	12.5 ml	25 ml
1% bis-acrylamide	3.15 ml	6.3 ml
1M Tris pH8.9	11.2 ml	22.4 ml
Glass Distilled Water	4.5 ml	9 ml
10% SDS	300 µl	600 µl
Ammonium Persulfate	100 µl	200 µl
Temed	30 µl	60 µl

Running Conditions: Constant current, 25 mA/gel for 4-5 hrs.

APPENDIX B

Protein Assay:

Biorad Micro Assay (1-20 μg protein, <25 μg/ml)

Bovine Serum Albumin (BSA) standards:

1. Stock made up at 1 mg /ml, and stored frozen.
2. Thaw and make dilute standard at 100 μg/ml protein by adding 0.5 ml stock to 4.5 mls distilled H_2O
3. Make up various concentrations of BSA to construct standard curve using the following dilutions:

Blank	dist.H_2O
2 μg/ml=1.6 μg	0.1 ml dil standard + 4.9 mls dH_2O
4 μg/ml=3.2 μg	0.2 ml dil standard + 4.8 mls dH_2O
8 μg/ml=6.4 μg	0.4 ml dil standard + 4.6 mls dH_2O
10 μg/ml=8 μg	0.5 ml dil standard + 4.5 mls dH_2O
20 μg/ml=16 μg	1.0 ml dil standard + 4.0 mls dH_2O

In a cuvette, add 0.8 ml of standard solutions or blank;

add 0.2 ml Dye concentrate.

Mix, and measure OD_{595} after 5 min to 1 hr.

For cell samples:

Typically CHO-SC1 and CHO-tsH1 cells have about 0.19 mg (or 190 μg) total protein/10^6 cells

For microassay: Add 50 μl of thawed cell suspension to 1.0 ml dH_2O, sonicate for 15 sec.

In a cuvette, add 0.8 ml sonicated cell sample to 0.2 ml of concentrated dye, mix and read OD_{595}.

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