

THE EFFECT OF HYPERTHERMIA ON THE RADIATION RESPONSE
OF CRYPT CELLS IN MOUSE JEJUNUM

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SUMMARY

The effect of hyperthermia and/or gamma-radiation on the survival of intestinal crypt cells was studied in BDF₁ mice using a microcolony assay. Hyperthermia treatments, which in themselves caused no detectable cell lethality, inhibited the capacity of crypt cells to repair sublethal radiation damage. In addition, heat applied either before or after single radiation exposures potentiated lethal damage to crypt cells; the degree of enhancement was dependent on the time interval between treatments. At the levels of heating employed, DNA synthesis in the intestinal epithelium was significantly reduced immediately following exposure, but returned rapidly to normal levels. No further disturbances in cellular kinetics were observed for up to 10 days after heating.

INTRODUCTION

Current investigations into the use of local hyperthermia in the treatment of cancer can be divided into two basic approaches. In one, the application of relatively high temperatures (generally well above the thermal tolerance of normal tissues) is used to bring about the thermal death of malignant cells. Experimental evidence indicating that hypoxic cells may be differentially sensitive to thermal killing (refs. 1 and 2) makes this approach attractive since it has been suggested that radioresistant hypoxic tumor cells may be an important factor in cases where conventional radiation therapy fails to produce local control. However, recent reports (ref. 3) indicate that in some cases cells may also be protected against heat by hypoxia. In addition, significant technical problems remain to be solved in confining heat to the tumor volume and in accurately monitoring temperatures in normal and tumor tissues in order to achieve a favorable therapeutic index under clinical conditions. The second approach is to utilize lower temperatures which in themselves do not cause cell death, but when combined with conventional treatment modalities, for example radiation or chemotherapeutic agents, increase their effectiveness. At the present time, this approach is technically more feasible in terms of clinical application.

Fractionated radiation therapy alone is effective in the local control of many human tumors. The therapeutic advantage in these instances is believed to involve differences between normal and tumor tissues with regard to such factors as repair of radiation damage, proliferative capability, redistribution throughout the cell cycle, and reoxygenation of hypoxic tumor cells. It has been cautioned (ref. 4) that if hyperthermia is to improve the local control of

tumors, the ideal combination should exploit the beneficial effects of heat without compromising the factors which are the basis of the existing therapeutic advantage of fractionated radiation therapy. Thus, to achieve this goal it is important to determine the effect of hyperthermia on each of these processes in normal as well as tumor tissue.

This paper reports the results of some preliminary experiments on the response of normal tissue to combined radiation and hyperthermia treatments. Specifically, the effect of hyperthermia on repair of radiation damage and the effect of treatment sequence and interval between treatments were studied. The crypt stem cell compartment of the mouse intestinal mucosa was chosen as the biological test system. This is a cell renewal system of the type that is dose limiting for the acute radiation response in clinical situations. In addition, crypt stem cells have a sizeable capacity to repair radiation damage (refs. 5 and 6) making them ideal for studies on the effect of hyperthermia on repair processes.

MATERIALS & METHODS

General

Adult female BDF₁ mice 8 to 12 weeks old weighing 20 to 22 grams were used in all experiments. Each treatment group consisted of 5 to 8 animals selected at random. Groups were caged separately and maintained in controlled temperature and lighting conditions throughout the experiments. Food and water were available ad libitum.

For hyperthermia treatments, unanesthetized animals were loosely restrained in thin-walled, 30mm x 82mm plastic tubes. The animals were immersed in an upright position in an insulated water bath to approximately mid-thorax level. The walls of the restraining tubes were perforated with numerous holes so that the animals were in direct contact with the water. A commercially available constant temperature circulator was used to exchange the bath. By adjusting the thermostat, heater wattage and flow rate, a variation of $\pm 0.1^{\circ}\text{C}$ from the desired bath temperature could be maintained throughout the treatments. In initial experiments, rectal temperatures were monitored during hyperthermia exposures using a recording thermistor.

Unanesthetized animals confined in a cylindrical lucite box received total-body gamma-irradiation at room temperature and in room air by using a conventional ⁶⁰Co teletherapy unit. The dose rate was 50 rads/min as determined by a Victoreen chamber placed in a mouse phantom positioned in the lucite holder.

Survival of intestinal crypt cells following radiation and/or heat treatments was determined by using the microcolony assay of Withers and Elkind (ref. 7). Three and one-half days after treatment, animals were killed by cervical dislocation. Segments of the jejunum were dissected out and fixed in buffered formalin. Histological slides of transverse sections taken at intervals along the length of the jejunum were prepared and stained with hematoxylin and eosin. Each section was examined microscopically and the number of crypts appearing around the circumference of the intestine recorded. Crypt survival for a given treatment was expressed as the average number of crypts per circumference

determined from counts of 4 to 5 sections from each of 3 to 8 animals.

Recovery (Repair) Experiments

Recovery from radiation damage was measured using the split-dose technique of Elkind and Sutton (ref. 8) and utilized by Withers and Elkind (refs. 5 and 6) in studies of the radiation repair capacity of intestinal crypt cells. In these experiments, animals were given two doses of radiation separated by varying intervals of time. Control animals received an equivalent dose as a single exposure. To test the effect of heat on repair of radiation damage, hyperthermia treatments were initiated as soon as possible (within 3 to 4 min) after the end of the first radiation dose. After the hyperthermia treatment, animals were returned to their cages at room temperature for various intervals before exposure to the second radiation dose.

Sequence-Interval Experiments

In experiments to test the effect of the order of hyperthermia and radiation treatments and the interval between treatments, animals were given an initial exposure of either heat or radiation then caged at room temperature for varying time intervals prior to the appropriate second treatment.

Effect of Heat on Stem Cell Kinetics

In order to examine the effect of hyperthermia treatments on crypt cell kinetics, animals were heated and at intervals up to 10 days after treatment, groups of 5 or 6 animals were injected intraperitoneally with 25 microcuries of tritiated thymidine ($^3\text{H-TdR}$). Thirty minutes later the animals were killed by cervical dislocation. Jejunal segments were then dissected out and immediately placed in iced, buffered saline. Sections approximately 5 mm in length were immediately slit longitudinally, rinsed in saline, blotted, and weighed to 0.1mg. The sections were then fixed overnight in acetic alcohol (1:3). The following day, sections were transferred to scintillation counting vials and solubilized at 55°C for 1 hr. Ten ml of scintillation cocktail were added and radioactivity determined in a liquid scintillation counter. Raw data were converted to dpm/mg wet weight of jejunum using quench correction data obtained from a quenched tritium standard set counted with the gut samples. Within certain limitations, these values are directly related to the number of crypt cells in the DNA synthetic phase (S-phase) of the cell cycle (ref. 9).

RESULTS AND DISCUSSION

Animals placed in the water bath for hyperthermia treatment required several minutes before their body temperature reached that of the bath. Figure 1 is a typical heating curve recorded from the rectum of an animal placed in a 42.1°C bath. Equilibration was obtained after 8 to 10 min. Once equilibration was achieved, it was maintained for the duration of the longest heating periods employed (30 min). Comparable results were observed at lower bath temperatures and with thermistors implanted directly in the lumen of the jejunum. A dis-

advantage of this method of heating is that the thermal tolerance of the whole animal is much lower than in techniques involving smaller heated volumes. For example, most of the animals heated for 30 min in a 42°C bath were moribund upon removal from the bath, and a significant proportion (up to one-third) died within 3 to 4 hr after exposure. The body temperature of these animals dropped rapidly to room temperature after removal from the bath; however, survivors regained their homeothermic capability by the following day. It has been reported that in mice heated under similar conditions, brain temperature may reach that of the bath even though the animal's head is well out of the water (ref. 10). Therefore, damage to areas of the hypothalamus responsible for integration of temperature control mechanisms may be involved. This post-heating response is almost certainly different from that following local heating techniques involving proportionately smaller tissue volumes. It must be considered as a possible factor in differences that may arise in the response of tissues to combination treatment under the two methods of heating. The dose response for thermal death as a function of bath temperature must increase very rapidly under the essentially whole-body heating conditions employed in these experiments since no early deaths were observed in animals heated for 30 min in 41.5°C baths; these animals rapidly regained their normal level of activity after heating.

The effect of hyperthermia on the capacity of crypt stem cells to repair radiation damage is shown in figure 2. The upper curve (circles) indicates the amount of recovery that occurred as a function of time between two radiation doses of 700 rads each. The crypt recovery factor plotted on the ordinate is the ratio of the number of crypts/circumference that survived the split dose (S_2) to the number of crypts/circumference that survived a single dose of 1400 rads (S_1). Maximum recovery, amounting to slightly more than a 5-fold increase in surviving crypts, occurred within about 4 hours. This split dose response is similar to those reported for a variety of biological systems. It is most often interpreted as indicating intracellular repair of sublethal damage inflicted by the first radiation dose (ref. 8), although other interpretations have been suggested (ref. 11).

The dashed curve (squares) traces recovery that occurred in animals heated at 42°C for 30 min immediately after exposure to 700 rads and then returned to room temperature for various times prior to a second 700 rad exposure. The maximum amount of recovery in heated animals was about 40% of that seen in animals held at room temperature between radiation treatments. No reduction in crypts was detected in animals receiving the hyperthermia treatment only. In animals receiving the second radiation dose immediately after the heat treatment, it was consistently observed that crypt survival was lower than in animals receiving the equivalent single dose. This resulted in a recovery factor less than one (square at one-half hr in fig. 2). This observation will be discussed below.

The effect of the length of the heat treatment on the extent of recovery is also shown in figure 2. The triangles show crypt recovery factors for animals irradiated with two 700 rad doses separated by 3 hours and treated immediately after the first exposure in a 42°C water bath. The numbers beside each symbol indicate the length of heat exposure in minutes. The hyperthermia treatment had to exceed 10 minutes before appreciable inhibition could be detected. This information is replotted in figure 3 as the percent inhibition of 3-hour recovery

as a function of heating time. After equilibration with the bath, inhibition of recovery increased rapidly with time reaching maximum value of about 60% inhibition at 30 min. Similar experiments were done to examine the influence of bath temperature on recovery (fig. 4). It is evident that recovery inhibition was highly temperature dependent and decreased from about 60% to less than 10% over a range of 2° C.

Reduced capacity to repair radiation damage after hyperthermia is a consistent finding in many biological systems. The denaturation of enzymes involved in the repair of radiation-induced lesions in DNA has been suggested as the mechanism involved in this inhibition (ref. 12). Unrepaired DNA damage may subsequently become lethal to the cell. Studies at the molecular level involving the effect of heat on repair of specific types of lesions (single strand breaks) support this hypothesis (ref. 13).

Results of experiments to examine the dependence of crypt survival on the sequence of combined hyperthermia-radiation treatments and on the time lapse between heat and radiation administration are shown in figure 5. The solid lines indicate crypt survival in animals heated at 41.5°C for 30 min at various times before or after a single radiation dose of 1200 rads. Crypt survival in animals which received radiation only (1200), heat only (41.5°) or neither treatment (C) is also indicated for reference. (The abscissa does not apply to these points.) When the interval between treatments was 2 hr or less, a hyperthermia treatment which was itself sublethal significantly enhanced the lethal effect of 1200 rads. Enhancement was achieved in both treatment sequences. The extent of potentiation was dependent on treatment interval, being greatest for no delay between exposures and decaying as the interval between treatments increased. The rate of decay of potentiation was nearly the same for heat applied before or after radiation; the loss of potentiation may have occurred at a slightly slower rate when heat followed radiation although further experiments must be carried out to confirm this. (In figure 5, compare the solid post-heat curve with the dashed curve representing the reversed pre-heat curve.) These time-dependent patterns of heat-radiation interaction in crypt cells differ from those reported for other biological systems. For example, heat potentiation of radiation damage in the skin of mice (ref. 14) and in cultured plateau phase mammalian cells (ref. 15) was found to decay more slowly when heat preceded radiation. In the latter case, it was suggested that repair of sublethal heat damage may be slower than repair of sublethal radiation damage. However, the mechanism of heat potentiation of radiation damage is not known. Although there is extensive evidence that heat can affect enzymatic radiation repair mechanisms, less attention has been given to the possibility that heat may modify either the radiation target itself or the initial radiation-induced lesions (ref. 12).

Heat potentiation of radiation damage in crypt cells (fig. 5) is probably the explanation for the consistent observation of a recovery factor less than one in split-dose experiments (fig. 2) in which both radiation doses and the intervening heat treatment were all given within a very short interval. In these cases, heat enhanced the effect of both the first and second radiation doses resulting in a lower level of crypt survival than that seen in animals receiving an equivalent single dose of radiation.

The data in figure 5 also suggest that as the time interval between treatments increased beyond 2 to 3 hr, heat may have protected crypt cells from radiation lethality. Crypt survival values for animals heated 2 and 4 hr before and 4 hr after irradiation were higher than for animals receiving radiation alone. The differences, however, are marginal and must be substantiated in further experiments.

The fact that the heat treatments employed in these experiments were sublethal in terms of crypt survival did not rule out the possibility that alterations in crypt stem cell kinetics might occur as a consequence of heating. Marked differences in the proliferative activity of the intestinal epithelium have been observed following exposure to radiation as well as chemotherapeutic agents (refs. 16, 17, 18, 19, and 20). In addition, it has been reported that cells in culture can be blocked at specific points in the cell cycle as a result of hyperthermia treatment (refs. 21 and 22). It should be assumed that such changes in cell kinetics could be important determinants of the response of renewal tissues to combined modality treatments. Thus, preliminary experiments were carried out to investigate the occurrence of altered crypt cell proliferation. Figure 6 shows the 30-min incorporation of ^3H -TdR into the DNA of intestinal crypt cells of mice heated for 30 min in a 42°C water bath as a function of time after treatment. Radioactivity (dpm/mg wet weight) of jejunal segments from heated animals is presented on the ordinate as a percent of control radioactivity of segments obtained from unheated animals. ^3H -TdR incorporation in this type of experiment is a direct measure of S-phase cellularity and is assumed to be indicative of the size of the proliferative compartment of the intestinal epithelium (ref. 9). The marked depression in ^3H -TdR incorporation which occurred in crypt cells sampled 1 hr after heating, however, was more likely a reflection of decreased DNA synthetic activity rather than a reduction in the number of S-phase cells. Recovery from this depression was rapid and the radioactivity of samples obtained from animals 12 hr to 10 days after heating fluctuated within $\pm 10\%$ of control values. Therefore, although short-term changes in the rate of DNA synthesis may have occurred after the heat exposures employed in the combined modality experiments reported here, marked long-term alterations in crypt stem cell kinetics were not detected.

The results of these experiments indicate that caution must be exercised in clinical situations where it is proposed to use mild heating as an adjunct to conventional radiation therapy. If heating of the tumor volume results in increased temperatures in surrounding irradiated normal tissues as well, radiation damage there may be significantly enhanced and its normal repair compromised. An increase in therapeutic advantage thus may not be realized in combined hyperthermia-radiation treatment as a consequence of these alterations in tissue response.

REFERENCES

1. Dewey, W.C.; Hopwood, L.E.; Sapareto, S.A.; and Gerweck, L.E.: Cellular Responses to Combinations of Hyperthermia and Radiation. *Radiology*, vol. 123, 1977, pp. 463-474.
2. Dewey, W.C.; Thrall, D.E.; and Gillette, E.L.: Hyperthermia and Radiation- A Selective Thermal Effect on Chronically Hypoxic Tumor Cells in vivo. *Int. J. Radiation Oncology Biol. Phys.*, vol. 2, 1977, pp. 99-103.
3. Bass, H.; Moore, J.L.; and Coakley, W.T.: Lethality in Mammalian Cells Due to Hyperthermia under Oxidic and Hypoxic Conditions. *Int. J. Radiat. Biol.*, vol. 33, 1978, pp. 57-67.
4. Harris, J.R.; Murthy, A.K.; and Belli, J.A.: Repair following Combined X-ray and Heat at 41°C in Plateau-Phase Mammalian Cells. *Cancer Research*, vol. 37, 1977, pp. 3374-3378.
5. Withers, H.R. and Elkind, M.M.: Dose-Survival Characteristics of Epithelial Cells of Mouse Intestinal Mucosa. *Radiology*, vol. 91, 1968, pp. 998-1000.
6. Withers, H.R. and Elkind, M.M.: Radiosensitivity and Fractionation Response of Crypt Cells of Mouse Jejunum. *Radiation Res.*, vol. 38, 1969, pp. 598-613.
7. Withers, H.R. and Elkind, M.M.: Microcolony Survival Assay for Cells of Mouse Intestinal Mucosa Exposed to Radiation. *Int. J. Radiat. Biol.*, vol. 17, 1970, pp. 261-267.
8. Elkind, M.M. and Sutton, H.: Radiation Response of Mammalian Cells Grown in Culture. I. Repair of X-ray Damage in Surviving Chinese Hamster Cells. *Radiation Res.*, vol. 13, 1960, pp. 556-593.
9. Hagemann, R.F.: Intestinal Cell Proliferation During Fractionated Abdominal Irradiation. *Brit. J. Radiol.*, vol. 49, 1976, pp. 56-61.
10. Hofer, K.G.; Choppin, D.A.; and Hofer, M.G.: Effect of Hyperthermia on the Radiosensitivity of Normal and Malignant Cells in Mice. *Cancer*, vol. 38, 1976, pp. 279-287.
11. Alper, T.: Elkind Recovery and "Sub-lethal Damage": A Misleading Association? *Brit. J. Radiol.*, vol. 50, 1977, pp. 459-467.
12. Bronk, B.V.: Thermal Potentiation of Mammalian Cell Killing: Clues for Understanding and Potential for Tumor Therapy. In *Advances in Radiation Biology*, vol. 6, Academic Press, 1976, pp. 267-324.
13. Corry, P.M.; Robinson, S.; and Getz, S.: Hyperthermic Effects on DNA Repair Mechanisms. *Radiology*, vol. 123, 1977, pp. 475-482.

14. Stewart, F.A. and Denekamp, J.: Sensitization of Mouse Skin to X-Irradiation by Moderate Heating. *Radiology*, vol. 123, 1977, pp. 195-200.
15. Harris, J.R.; Murthy, A.K.; and Belli, J.A.: The Effect of Delay Between Heat and X-Irradiation on the Survival Response of Plateau Phase V-79 Cells. *Int. J. Radiation Oncology Biol. Physc.*, vol. 2, 1977, pp. 515-519.
16. Leshner, S.: Compensatory Reactions in Intestinal Crypt Cells after 300 Roentgens of Cobalt-60 Gamma Irradiation. *Radiation Res.*, vol. 32, 1967, pp. 510-519.
17. Hagemann, R.F.; Sigdestad, C.P.; and Leshner, S.: Intestinal Crypt Survival and Total and Per Crypt Levels of Proliferative Cellularity Following Irradiation: Single X-ray Exposures. *Radiation Res.*, vol. 46, 1971, pp. 533-546.
18. Hagemann, R.F. and Concannon, J.P.: Mechanism of Intestinal Radiosensitization by Actinomycin D. *Brit. J. Radiol.*, vol. 46, 1973, pp. 302-308.
19. Burholt, D.B.; Hagemann, R.F.; Schenken, L.L.; and Leshner, S.: Influence of Adriamycin and Adriamycin-Radiation Combination on Jejunal Proliferation in the Mouse. *Cancer Res.*, vol. 31, 1977, pp. 22-27.
20. Ho, E.; Correy, C.; and Maruyama, Y.: Enhancement of Radiation Effect on Mouse Intestinal Crypt Survival by Timing of 5-Fluorouracil Administration. *Radiology*, vol. 125, 1977, pp. 531-532.
21. Schlag, H. and Lucke-Huhle, C.: Cytokinetic Studies on the Effect of Hyperthermia on Chinese Hamster Lung Cells. *Europ. J. Cancer*, vol. 12, 1976, pp. 827-831.
22. Dewey, W.C. and Highfield, D.P.: G_2 Block in Chinese Hamster Cells Induced by X-Irradiation, Hyperthermia, Cycloheximide, or Actinomycin-D. *Radiation Res.*, vol. 65, 1976, pp. 511-528.

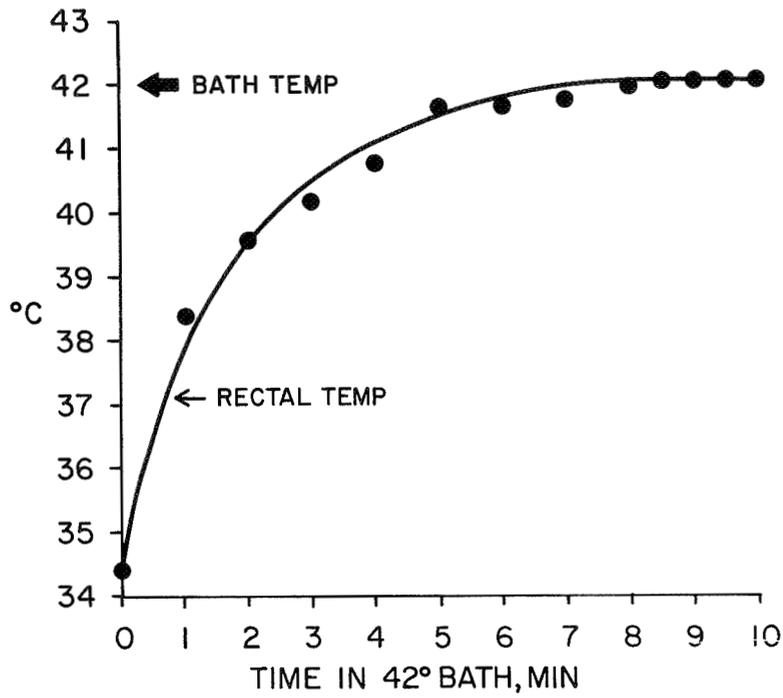


Figure 1.

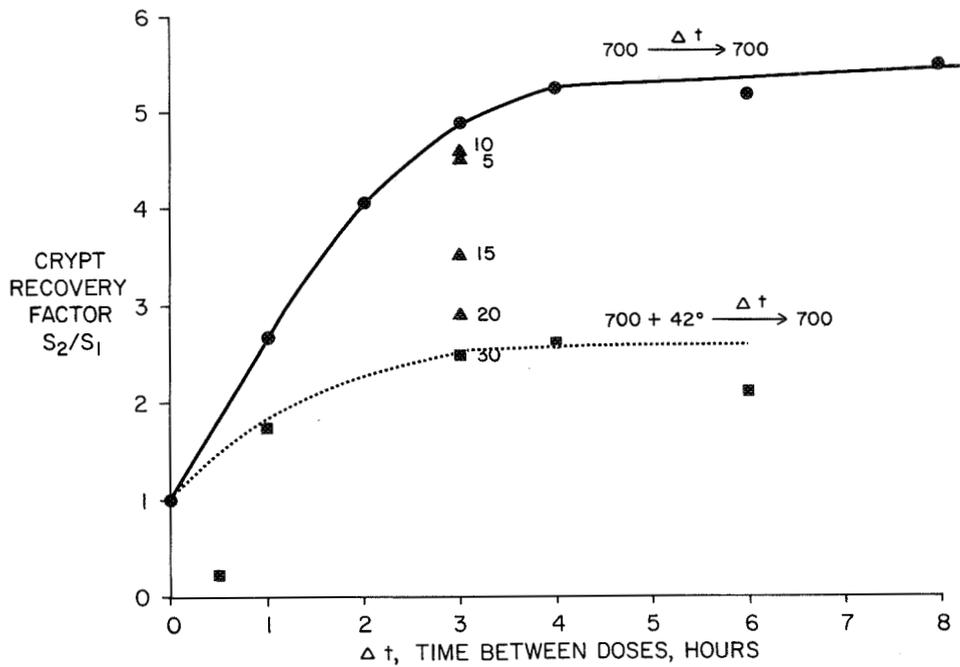


Figure 2.

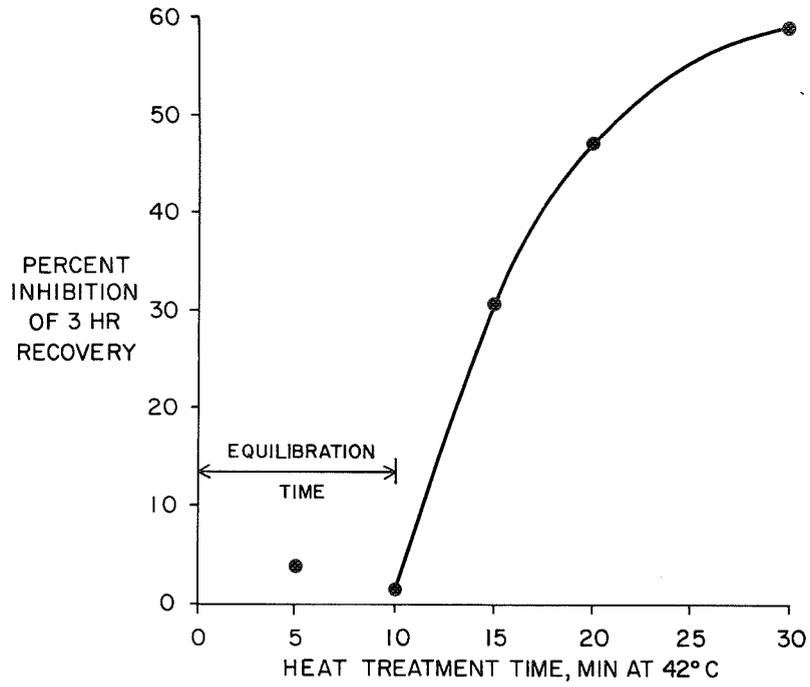


Figure 3.

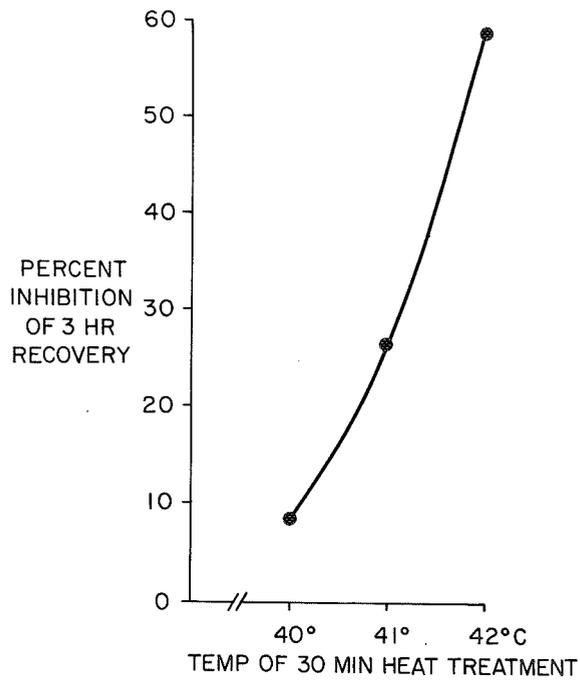


Figure 4.

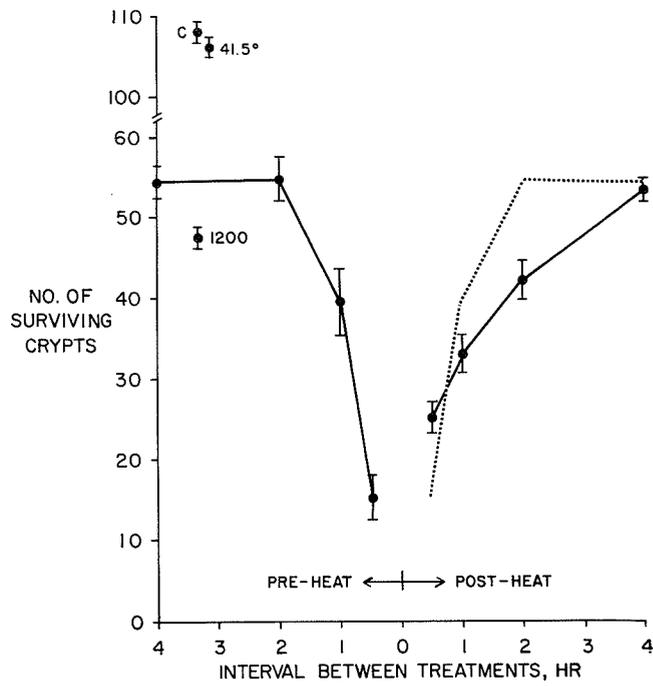


Figure 5.

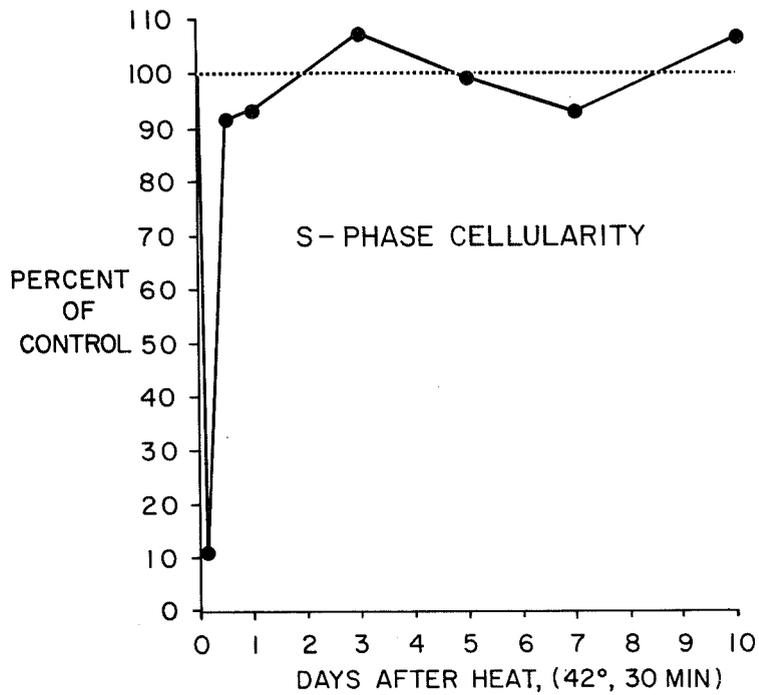


Figure 6.