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AS A POSSIBLE RATE LIMITING STEP
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ABSTRACT

THE DENATURATION OF DNA REPAIR PROTEINS
AS A POSSIBLE RATE LIMITING STEP
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The experiments to be described support the hypothesis that the denaturation of DNA repair proteins is a rate limiting step in the thermal death of cells grown in tissue culture. Mammalian fibroblasts were subjected to small changes in temperature before ultraviolet (UV) irradiation in order to detect changes in repair of UV induced damage. Confluent fibroblasts were incubated for three days in a medium deficient in arginine and isoleucine. After this period the cells were incubated for one hour with hydroxyurea and then treated with 254 nm UV radiation at various doses. During various spans of time immediately before UV treatment, the cells were incubated at different temperatures. Repair of DNA was measured by several methods after UV treatment.

In some experiments repair was measured by incorporating ^{3}H-Thymidine at 37°C for different lengths of time after UV irradiation. Human skin and hamster (V-79) fibroblasts incubated at either 40°C or 41°C for 3 days immediately prior to UV treatment showed significantly less ^{3}H-Thymidine incorporation into their DNA than cells that were constantly incubated at 37°C prior to

UV treatment. This result was observed regardless of the length of the period of ^{3}H-Thymidine incorporation. Cells incubated at 33°C prior to UV treatment did not differ significantly from cells that were always incubated at 37°C in their incorporation of ^{3}H-Thymidine.

Autoradiography showed that V-79 cells incubated at 41°C prior to UV treatment had a fairly wide distribution of grains per nucleus, but with average number of grains significantly less than the average number of grains in cells always incubated at 37°C. This suggests that prior heat treatment impairs the repair in all of the cells rather than causes the thermal death of a large portion of the cells.

Finally, an endonuclease specific assay was conducted to determine whether the above effects were due to the thermal denaturation of the endonuclease repair enzyme or rather to some heat sensitive process involved in the transport and phosphorylation of exogenous ^{3}H-Thymidine to ^{3}H-dTTP into the DNA inside the cells. By using a dimer specific endonuclease from M. luteus, the number of dimers produced and repaired was measured directly. For both hamster and human fibroblasts, significantly less repair was observed in cells incubated at 41°C prior to UV treatment than in cells incubated at 37°C before UV treatment. This suggests that the endonuclease repair enzymes are denatured at the higher temperature. The evidence shows that the thermal denaturation of the endonuclease is the rate limiting step for one of the processes leading to the death of V-79 cells.

To my parents

George and Stella Shenkar

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CHAPTER I

INTRODUCTION

The experiments that are to be described were conducted to test the hypothesis that the denaturation of DNA repair proteins is the rate limiting step in the thermal death of single cells. This hypothesis arose from the evidence presented by Rosenberg and coworkers[1] that protein denaturation is the cause of thermal death in living systems; and the correlation between maximum life span of mammals and extent of repair of DNA damaged by ultraviolet radiation as described by Hart and Setlow[2].

PROTEIN DENATURATION AS THE CAUSE OF THERMAL DEATH

It is well known that temperature affects biological systems in a very dramatic manner. For example, at temperatures higher than 315°K, most proteins denature with pseudo-first order kinetics since the renaturation rate is negligible[3]. The rate is described by

$$dn/dt = -k_D n \qquad (1),$$

where k_D is the first order denaturation constant and is expressed by the absolute rate theory equation[4] as

$$k_D = \kappa \frac{k_B T}{h} e^{\Delta S^{\dagger}/R} e^{-\Delta H^{\dagger}/RT} \qquad (2).$$

The transmission coefficient, κ, is taken to be equal to unity; and k_B, h, and R are the Boltzmann, Planck, and gas constants,

respectively. The activation enthalpy, $\Delta H^{\dagger}$, and the activation entropy, $\Delta S^{\dagger}$, for thermal denaturation of proteins follow a compensation law[5-9],

$$\Delta S^{\dagger} = \frac{1}{T_c} \Delta H^{\dagger} + b \qquad (3),$$

where T_c and b are constants. Furthermore, the thermal death of viruses[10], bacteria[11-13], yeasts[14], and mammalian cells in tissue culture[15-19] obey the same compensation law with the same constants: $T_c = 325°K$ and $b = -64.5$ cal/mol °K. These observations led to the hypothesis that protein denaturation is the rate limiting step in the thermal death of single cells.

The life span of multicellular poikilotherms also has a strong temperature dependence. Most of these organisms show senescense, an increased probability of dying with age. The first mathematical expression of survivorship curves of multicellular organisms was reported by Gompertz (1825)[20]:

$$\mu = -\frac{1}{N}\frac{dN}{dt} = R_o e^{\alpha t} \qquad (4).$$

The rate of mortality, μ, is expressed by a time dependent function containing two constants R_o and α. The power law has recently been applied to the survivorship of multicellular organisms[1]:

$$\mu(t) = -\frac{1}{N}\frac{dN(t)}{dt} = At^{n-1} \qquad (5),$$

or

$$\frac{N(t)}{N_o} = e^{-At^n/n} \qquad (6),$$

where A and n are constants.

The power law has several advantages over the Gompertz law and its modifications[1]. Survivorship curves for many multicellular organisms are better fitted by the power law than by the Gompertz function. Furthermore, the power law contains only two constants. Although the Gompertz function also contains two constants, its modifications by Makeham[21] and the addition of Perk's function[22], which provide a better fit to the data, contain additional constants. Only one temperature dependent constant is contained in the power law, while both constants are temperature dependent in the Gompertz function. The logarithm of A, the temperature dependent constant of the power law, plotted against the reciprocal temperature yields an Arrhenius plot. The activation enthalpy for death can be obtained from the slope of this Arrhenius plot. The activation enthalpy cannot be obtained from the Gompertz function. Finally, the number of rate limiting steps is n + 1, where n is the temperature independent constant of the power law. The number of rate limiting steps cannot be obtained from the Gompertz function.

The power law can be used in a thermodynamic analysis of death because of these advantages over the Gompertz function. For example, it can be applied to Drosophila melanogaster. From the survivorship plots of ln ln (N_o/N) against ln t, the constants A and n can be obtained as a function of temperature[1]. Over a small temperature range (25°C - 33°C), n is found to vary from 5.2 to 5.8, or n = 5.5 $\pm$ 6.6% for Drosophila[1]. Thus n is fairly temperature independent. The number of rate limiting steps, n + 1, for the death of Drosophila is then probably 6 or 7. The values of A vary by four orders of magnitude over the range 25°C - 33°C[1]. Finally, the plot of log A versus the reciprocal absolute

temperature yields a good straight line[1]. This is an Arrhenius plot with the equation,

$$A = A_o e^{-\Delta H^{\dagger}/RT} \qquad (7).$$

The activation enthalpy for the mortality rate is obtained from the slope of the Arrhenius plot. The activation enthalpy for death in Drosophila melanogaster is 190 kcal/mol[1].

The sum of the activation enthalpies of the rate limiting steps for the mortality rate is equal to the activation enthalpy for death of a multicellular organism. This is a consequence of the sequential chain model for aging from which the power law can be derived[23]. The pertinent details are shown below.

The sequential model for aging consists of an abstract chain containing m identical but independent links[23]. A link is intact when in state 1,2,...n. A direct transition from state j is only permitted to state j + 1 (j = 1,2,...n). The transition probabilities, ρ, are assumed to be equal and independent of time. Let $p_j(t)$ be the probability that a given link is in state j at time t. The deterioration of the link can be described by the equations

$$\frac{\partial}{\partial t} p_j(t) = \rho[p_{j-1}(t) - p_j(t)] \qquad (8),$$

where j = 1,2,...n,

$$p_0(t) = 0 \text{ for all } t \qquad (9),$$

$$p_j(0) = 1;\ j = 1$$

$$= 0;\ j \neq 1 \qquad (10).$$

If $f_j(s)$ is the Laplace transform of $p_j(t)$,

$$f_1(s) = \frac{1}{s+\rho} \qquad (11),$$

$$f_j(s) = \frac{\rho}{s+\rho} f_{j-1}(s) = \frac{\rho^{j-1}}{(s+\rho)^j} \qquad (12),$$

for $j = 1,2,\ldots n$. Taking the inverse transform of $f_j(s)$,

$$p_j(\rho t) = e^{-\rho t}(\rho t)^{j-1}/(j-1)! \qquad (13),$$

for $j = 1,2,\ldots n$.

When any link undergoes the transition from state n to state n + 1, it will break, and the organism will perish. The probability that the link is still intact at time t is

$$F(t) = \sum_{j=1}^{n} p_j(t) \qquad (14).$$

Therefore,

$$F(\rho t) = e^{-\rho t} \sum_{j=1}^{n} \frac{(\rho t)^{j-1}}{(j-1)!} \qquad (15).$$

For $(\rho t) << 1$,

$$F(\rho t) \sim 1 - \frac{(\rho t)^n}{n!} \qquad (16),$$

and the probability that the chain is still unbroken is

$$S(t) = F^m(t) = \left(\sum_{j=1}^{n} p_j(t) \right)^m \qquad (17).$$

The age-specific failure rate, the mortality rate, is expressed as,

$$\mu(t) = \frac{1}{S(t)} \frac{dS(t)}{dt} = - \frac{mdF(t)}{F(t)dt} \qquad (18).$$

Substituting (16) into (18) and approximating,

$$\mu(\rho t) \sim m\rho(\rho t)^{n-1}/(n-1)! \qquad (19).$$

Thus,

$$\ln \mu = n \ln \rho + \ln [mt^{n-1}/(n-1)!] \qquad (20).$$

The only temperature dependent quantity on the right hand side of Equation (20) is ρ. By the theory of rate processes this dependence is given as

$$\rho = \rho_o \exp(-\Delta h^{\dagger}/RT) \qquad (21).$$

where $\Delta h^{\dagger}$ is the activation enthalpy corresponding to an individual, molecular level state transition, R is the gas constant, and T is the absolute temperature. Equation (20) becomes

$$\ln \mu = K - \frac{n\Delta h^{\dagger}}{RT} \qquad (22),$$

where $K = \ln[\rho_o^n m t^{n-1}/(n-1)!]$. The quantity $n\Delta h^{\dagger}$ is the "effective" activation enthalpy determined at the macroscopic level from the experimental data. Comparing Equations (5) and (7) with Equation (22) shows that the sequential chain model is actually a derivation of the power law:

$$\mu(t) = At^{n-1} = A_o e^{-\Delta H^{\dagger}/RT} t^{n-1} = [\rho_o^n m/(n-1)!] e^{-n\Delta h^{\dagger}/RT} t^{n-1} \qquad (23).$$

The constant $A_o = \rho_o^n m/(n-1)!$, while the activation enthalpy for death equals the sum of the activation enthalpies for the rate limiting steps, or $\Delta H^{\dagger} = n\Delta h^{\dagger}$. It was already stated that the activation enthalpy for the death of Drosophila melanogaster was 190 kcal/mol and the value of the constant n was $5.5 \pm 6.6\%$[1]. According to the sequential chain model, the activation enthalpy corresponding to an individual state transition, $\Delta h^{\dagger}$, would be approximately 34.5 kcal/mol. The fact that the activation enthalpies of the individual steps sum

in this manner is not the consequence of any physical or chemical characteristic of the underlying molecular process; it is due, instead, to statistics which sample the extreme rather than average properties of a system[23]. Although it was assumed here that the activation enthalpies, $\Delta h^{\dagger}$, for the individual steps were equal, they must be in fact at least roughly equal in the sequential chain model. It was shown[23] that if the activation enthalpy of one step was significantly greater than the activation enthalpy of the other steps, then the resulting survivor curves would be inconsistent with the experimental data[1] for Drosophila.

If the hypothesis that protein denaturation causing thermal death in single cells[3] is extended to the sequential chain model for multicellular organisms, the death of a multicellular organism should involve n + 1 protein denaturations each with activation enthalpy of about $\Delta H^{\dagger}/n$, where n and $\Delta H^{\dagger}$ are obtained from the survivorship curves. For example, the death of Drosophila should involve six or seven protein molecules each having activation enthalpies of about 34.5 kcal/mol for thermal denaturation.

THE CORRELATION BETWEEN THE MAXIMUM LIFE SPAN OF MAMMALS AND REPAIR OF DNA DAMAGED BY ULTRAVIOLET RADIATION[2]

The accumulation of damage in the DNA of cells increases with the age of the organism from which the cells were taken. Damage in tissues from aging mice was assayed by measuring with autoradiography the ability of DNA from these tissues to act as primers for in vitro nucleotide incorporation catalyzed by calf thymus polymerase[24,25]. The result that DNA from old tissues acted as better primers than from young ones indicated that DNA from old cells contains relatively large numbers of strand breaks.

Measurements of alkali-labile bonds (single-strand breaks) in the DNA of old and young cells supported this interpretation. Since chick red cells did not repair x-ray breaks in their DNA, the DNA of chick red cells had more breaks than that from lymphocytes[26]. Furthermore, the DNA from old red cells had more breaks than the DNA from young ones. This same study also showed that old rat muscle cells did not repair x-ray breaks as well as young ones[26]. However, nondividing cells, such as rabbit retinal[27] or dog neuronal cells[28], were able to repair ionizing radiation breaks as well as fibroblasts.

Furthermore, rat retinal ganglion cells treated with the chemical carcinogen 4-nitroquinoline 1-oxide *in vitro* showed no age-associated changes in the levels of DNA repair measured by unscheduled DNA synthesis[29]. On the other hand, singly isolated beating heart cells from newborn rats performed unscheduled DNA synthesis after ultraviolet irradiation, while this same repair synthesis could not be induced in beating cardiac cells isolated from adult rats[30]. Also there was a decrease in DNA repair capacity during successive subcultures of primary abdominal rat fibroblasts treated with 4-nitroquinoline 1-oxide[31]. Not only did the capacity for excision repair of ultraviolet radiation damage to DNA in primary cultures of mouse embryonic cells decrease with successive transfers *in vitro*, but also this repair capacity decreased in cells taken from later stages of development[32]. Moreover, hepatic cells of old mice were able to rejoin gamma radiation-induced DNA scissions as quickly as those of young mice, but only the radiation-induced DNA breaks, and not the age-associated scissions, were rejoined[33]. It was also shown that frequencies of mitomycin-C induced sister

chromatid exchanges declined with age in mouse and rat bone marrow cells in vivo suggesting an altered response to DNA damage with aging[34].

The premature aging syndromes of Hutchinson-Gilford (progeria), Werner, and Rothmund Thompson are thought to be repair deficient[35-37]. There is evidence of abnormal enzyme structure/function in cells with these premature aging syndromes which seem to mimic the normal cells when they senesce[38,39]. Although it was shown that fibroblasts isolated from patients suffering from progeria were deficient in the ability to repair DNA strand breaks induced by gamma radiation[40], repair replication was normal after ultraviolet irradiation in the progerioid cells[41,42].

Although these studies seem to indicate that there is a general decline in DNA repair with the age of the animal, there might not necessarily be an age-related decline in DNA repair of a specific cell type. Furthermore, a correlation was observed between DNA repair and maximum life span of an animal.

In one study fibroblasts from seven mammalian species that had completed about one twentieth of their life span were subjected to several fluences of ultraviolet radiation and then allowed to incorporate ^{3}H-Thymidine for various periods of time[2]. The ability of the cells to perform unscheduled DNA synthesis (a measure of excision repair) was measured by autoradiography. Both the initial rate and the maximum incorporation of ^{3}H-Thymidine increased with the maximum life span of the species (shrew, mouse, rat, golden hamster, cow, elephant, and human). The maximum extent of unscheduled

DNA synthesis was approximately proportional to the logarithm of the maximum life span of the species.

Investigators who have employed ultraviolet irradiation to human cells in tissue culture agree that, although there is an age related decline in the cells' ability to perform repair synthesis, this decline is not the primary cause of cellular aging and eventual death[43-46]. However, these same studies disagree as to when this failure becomes evident as the cells are progressively subcultured. Some studies have indicated that this failure does not become apparent until very late in the in vitro life span[43], possibly not until the last passage[44]. Other studies indicated that human diploid fibroblasts exhibit a reduced capacity to repair damaged DNA relatively early in their life span[45,46]. Major decreases were observed in DNA repair of WI-38 cells after approximately 60% of their life span had been completed[46]. There was also a strong correlation between cells unable to carry out semi-conservative DNA synthesis for division and those unable to perform repair synthesis[46]. A more recent study measured unscheduled DNA synthesis after ultraviolet irradiation in both confluent and arrested human diploid fibroblasts[47]. In this study confluent cultures exhibited identical levels of unscheduled DNA synthesis at all in vitro ages. Cells arrested by lowering the serum concentration of the incubation medium exhibited similar levels of excision repair as did confluent cells during the initial one-third of the cells' characteristic in vitro life span. However, the arrested cells exhibited a 30% to 50% increase in the amount of detectable DNA repair during the final two-thirds of the life span.

Certainly there is much more to aging than just the failure of an excision repair system for the removal of ultraviolet radiation-induced pyrimidine dimers. In some systems excision repair of ultraviolet induced dimers has been shown to be fairly independent of the overall aging process. Individuals with xeroderma pigmentosum are defective in excision repair[41,48,49], have skin abnormalities[50], and develop potentially lethal skin cancer if exposed to sunlight[51]. Some of these individuals also have ocular, nervous, or endocrine disorders and often their life span is decreased[50]. However they do not seem to age in other ways or have a life span shorter than a mouse, despite observations that cells from these individuals do less excision repair than mouse fibroblasts after ultraviolet irradiation[2,41,48,49]. Also xeroderma pigmentosum fibroblasts do not go through less passages than normal ones in culture[43,52]. On the other hand, fibroblasts from individuals who age prematurely (progeria) do not go through as many passages as do normal cells[53,54], but show normal levels of excision repair[41,42].

Furthermore, a correlation between DNA excision repair and life span does not necessarily prove that less DNA excision repair causes aging. There are also correlations between life span of mammals and other parameters (brain weight, body weight, and metabolic rate)[55]. However there is no evidence that any of these parameters determines the life span of a mammal. It is possible that the failure of any cellular repair process does not cause, but rather is a consequence of, the aging of a multicellular organism. Certainly it is very improbable that the amount of excision repair of pyrimidine dimers induced by ultraviolet irradiation is the most important determinant

of the maximum life span of a mammal, because there will always be some damage to DNA that will not be repaired via an error-free excision process even in normal cells. It is more likely that the amount of excision repair of ultraviolet radiation-induced pyrimidine dimers is only a correlate of the maximum life span potential of an organism. A multicellular organism might have many processes that are tuned so that all of them fail at the time the organism senesces.

Nevertheless, the study of the excision repair of dimers in DNA may be very important in understanding the aging process. First of all, excision repair of pyrimidine dimers is the only molecular repair process discovered to date that has been correlated with the maximum life span of an organism[2]. This is certainly more significant than the correlations with gross parameters such as brain and body weight. The significance is that the rate of the aging process and the maximum life span of a multicellular organism may be determined by its individual cells, since each cell carries information that enables an investigator to predict the life span of the organism from which it was taken by its extent of excision repair of ultraviolet radiation-induced dimers. Moreover, defects in excision repair may cause aging and death in individual cells. Certainly, unrepaired damage of DNA could be detrimental to the cell[2]. Furthermore, the excision repair system in cells most likely involves proteins. These repair proteins probably thermally denature, causing less overall repair in the cells. A thermodynamic analysis can be applied to these repair protein denaturations. Finally, the observation that excision repair capacity decreased in mouse embryonic cells taken from later

stages of development[32] suggests that the amount of repair is influenced by cellular differentiation[56].

THE HYPOTHESIS THAT DENATURATION OF DNA REPAIR PROTEINS IS THE RATE LIMITING STEP IN THE THERMAL DEATH OF SINGLE CELLS

The compensation law (3) involving protein denaturation and thermal cell death leads to the hypothesis that protein denaturations are the rate limiting steps in the death of single cells[3]. The observations that both DNA damage and cell death increase with the passage number of the cells in tissue culture[43-46] or the age of the animal from which the cell was taken[24-42], and the correlation between life span and DNA repair[2], lead to the hypothesis that decreased DNA repair causes cell death. This would be a composite decrease of all DNA repair, which may include, but not necessarily be restricted to, the excision repair of ultraviolet radiation-induced pyrimidine dimers. From these two hypotheses it follows that thermal denaturation of repair proteins may be the rate limiting step in the death of single cells.

If the hypothesis that repair protein denaturation causing cell death is correct, then repair protein denaturation would be an activated process and a thermodynamic analysis could be applied to it. It would then be expected that a small increase in temperature would cause a large number of repair proteins to denature, resulting in a large decrease in repair. However, there are at least five possibilities for this expected observation with a small increase in temperature: (1) repair proteins may denature and repair ineffectively if not at all, (2) the manufacturing rate of the repair proteins may decrease, (3) repair proteins may convert into damaging proteins, (4) processes which damage may have their rates increased more than

the rate of repair, and (5) proteins in close proximity to the repair site may denature and interfere with the repair process. It will be shown in Chapter V (p. 158) that only the first, (1), and the last, (5), of these possibilities are consistent with the results of the experiments that were conducted in the present investigation.

There may be many causes of the thermal death of single cells other than repair protein denaturation. For example, the denaturation of a lysosomal protein might cause a leak in a lysosome, allowing a toxic substance, contained in the lysosome, to escape and kill the cell. Even if repair protein denaturation is not the sole cause of cell death, or even the most important cause, the study of repair protein denaturation is important in understanding cellular aging and death. Assuming that there were other causes of cell death and these causes could somehow be eliminated, it would be unlikely that repair protein denaturation could be prevented. Eventually cell damage would accumulate, leading to cell malfunction, and would ultimately result in cell death. Furthermore the mechanism of repair protein denaturation leading to cell death may be similar to other causes of cell death.

EXPERIMENTS THAT WERE CONDUCTED IN ORDER TO SUPPORT THE HYPOTHESIS THAT DENATURATION OF DNA REPAIR PROTEINS IS A RATE LIMITING STEP IN THE THERMAL DEATH OF SINGLE CELLS

There are many repair mechanisms within a cell. For example, DNA can be damaged by ultraviolet irradiation, x-rays, gamma rays, and chemicals, and can be repaired by different mechanisms. It is not certain if any of these mechanisms are important for determining the life span of an organism.

However the measurement of repair of damage caused by ultraviolet radiation has several advantages over the measurement of repair caused by other damages. First of all the effects of ultraviolet irradiation on DNA can be well quantitated. Furthermore, the excision repair of DNA damaged by ultraviolet radiation has been analyzed by a number of different techniques[57-61], including the actual removal of cyclobutyl pyrimidine dimers (excision), repair replication (the incorporation of radioactive label into parental DNA during repair), unscheduled DNA synthesis (the synthesis of DNA during non-S periods of the cell), the photolysis of bromodeoxyuridine incorporated into parental DNA during repair, and endonuclease site specificity (the measurement of single strand breaks in the DNA at dimer sites). Finally, only repair of DNA damaged by ultraviolet irradiation has been correlated with the life span of an organism[2].

The experiments that were conducted measured DNA repair of damage caused by ultraviolet irradiation by the techniques of unscheduled DNA synthesis, autoradiography, and endonuclease site specificity. In the experiments utilizing unscheduled DNA synthesis, confluent mammalian fibroblasts were incubated for three days at conditions unfavorable for semi-conservative DNA replication. The cells were then exposed to ultraviolet radiation and then allowed to incorporate ^{3}H-Thymidine. The cells were incubated at different temperatures for various periods immediately before ultraviolet irradiation. Excision repair or unscheduled DNA synthesis was quantitated by the amount of ^{3}H-Thymidine incorporated during

repair. Less ^{3}H-Thymidine incorporation was observed with increased incubation temperature before ultraviolet irradiation.

The experiments involving autoradiography were identical to the unscheduled DNA synthesis experiments, except that excision repair was quantitated by tracks on a photographic emulsion. This experiment was performed to show that the decreased excision repair with increased pre-UV incubation temperatures that was observed in the unscheduled DNA synthesis experiments was due to decreased excision repair in all the cells rather than to the thermal death of a large portion of the cells.

The experiments involving endonuclease site specificity were identical with the other two experiments except that the cells were radioactively labeled before confluence, instead of during repair. After repair, the DNA was extracted from the cells and treated with an endonuclease from M. luteus. The endonuclease makes single strand breaks in the DNA at the ultraviolet induced dimer sites. Then by ultracentrifuging the DNA on sucrose gradients, the number of dimers could be quantitated. This experiment was done to show that increased incubation temperature before ultraviolet irradiation thermally impaired the endogenous excision repair mechanism, rather than hinder the transport and phosphorylation of exogenous ^{3}H-Thymidine into ^{3}H-dTTP in the DNA inside the nucleus of the cells.

CHAPTER II

THE EFFECT OF TEMPERATURE ON THE REPAIR OF ULTRAVIOLET INDUCED PYRIMIDINE DIMERS IN THE DNA OF MAMMALIAN CELLS AS MEASURED BY UNSCHEDULED DNA SYNTHESIS

INTRODUCTION

"Unscheduled DNA synthesis" induced by ultraviolet radiation was first demonstrated in HeLa cells[62]. The enzymatic "excision repair" of ultraviolet radiation induced pyrimidine dimers in DNA was later shown in HeLa cells[63]. Actual "repair replication" or "non-semiconservative DNA synthesis" had been reported in HeLa cells after treatment with ultraviolet radiation[64,65], x-rays[66,67], or alkylating agents[68]. "Unscheduled DNA synthesis" has been correlated with "repair replication" in several mammalian cell lines after ultraviolet irradiation[69,70]. Repaired DNA following ultraviolet irradiation can serve as a template for normal DNA synthesis[65,71]. Also in mammalian cell DNA, pyrimidine dimers and N-acetoxy-2-acetylaminofluorine lesions are substrates for excision repair enzymes[63,72-75].

Hydroxyurea can inhibit "semi-conservative DNA synthesis"[76-79] without affecting "excision repair"[63] or "repair replication"[80]. It has commonly been used to reduce the amount of normal "semi-conservative DNA synthesis" in order to detect the otherwise masked amounts of "unscheduled DNA synthesis" caused by damaging DNA with various agents[81-87]. Similarly, cells maintained in a medium deficient in

arginine also decreased the amount of "semi-conservative DNA synthesis"[88] enabling measurement of "unscheduled DNA synthesis"[89].

Hydroxyurea allows the detection of 5-fold to 9-fold increases in ^{3}H-Thymidine incorporation ("unscheduled DNA synthesis") in the essentially non-dividing human lymphocyte system following treatment with chemical carcinogens[82]. It is less effective in normally rapidly dividing cells[90]. Because "repair replication" appears to occur in the absence of protein synthesis in both HeLa and Chinese hamster cells[91], a sensitive assay was developed for quantitating "unscheduled DNA synthesis" in rapidly dividing eukaryotic cells by combining the two methods of inhibition of "semi-conservative DNA synthesis"[92]. When transformed human amnion (AV_3) cells were maintained in a culture medium containing hydroxyurea and deficient in arginine, a 10-fold to 20-fold increase in "unscheduled DNA synthesis" was measured following treatment with ultraviolet radiation of N-acetoxy-2-acetylaminofluorine[92]. This technique would enable the detection of different "DNA excision repair synthesis" after ultraviolet irradiation with small differences in the temperature of incubation before ultraviolet irradiation.

MATERIALS AND METHODS

Cells

Chinese hamster lung fibroblasts (V-79) and normal human skin fibroblasts (736 NF) were used in the experiments. Both cell cultures were obtained from J. E. Trosko, Human Development Department, Michigan State University, East Lansing, MI., and were grown under humidified 5% CO_2 in air.

Media

Permanent stock cultures of the Chinese hamster cells utilized a "C-15" medium (see Appendix A) supplemented with 5% fetal calf serum. Permanent cultures of human fibroblasts utilized a "D" medium (see Appendix A) supplemented with 10% fetal calf serum. Growing cell cultures for experiments utilized "D" medium supplemented with 5% fetal calf serum for V-79 cells and 10% fetal calf serum for human fibroblasts. The confluent cells utilized for experiments were maintained in "D" medium without arginine or isoleucine (see Appendix A) and supplemented with 5% dialysed fetal calf serum for V-79 cells and 10% dialysed fetal calf serum for human fibroblasts. All media were supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and mycostatin (100 units/ml).

Experimental Culture Procedure (Figure 1)

Cells were inoculated into either 60 mm plastic Petri dishes or in 25 cm^2 plastic flasks (Falcon Co., Oxnard, Calif. or Corning Glass Works, Corning, NY) and allowed to grow in "D" medium to heavy confluent densities. This took between 1 to 4 days for V-79 cells and between 1 to 2 days for human fibroblasts. At confluence, the "D" medium was replaced with a "D" medium deficient in arginine and isoleucine supplemented with dialysed fetal calf serum (5% for V-79 cells, 10% for human fibroblasts). After 24 hours, this medium was decanted and replaced with fresh "D" medium deficient in arginine and isoleucine, supplemented with the same concentration of dialysed fetal calf serum, and incubated for 48 hours. At this point, hydroxyurea was added to a final concentration of 5 mM. If used,

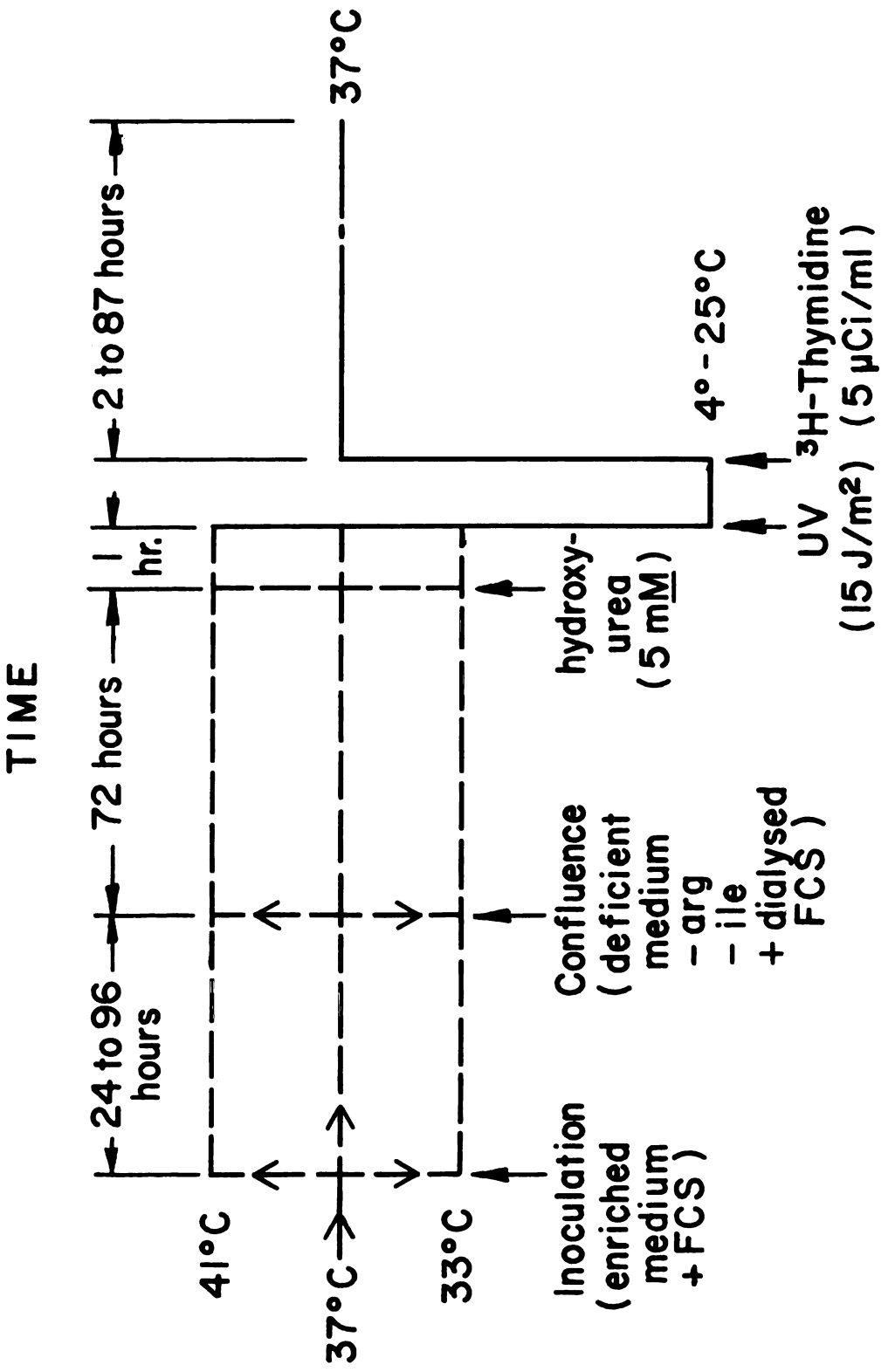


Figure 1. Experimental Culture Procedure

cycloheximide was added at this point to a final concentration of 5 μg/ml. Ultraviolet irradiation of the cells was done after one hour of incubation in the presence of hydroxyurea. Cells were incubated at various temperatures ranging from 33°C to 43°C for various spans of time up to 5 days immediately prior to ultraviolet irradiation, through some or no portion of the growing period in "D" medium, and through some or no portion of the confluent period in "D" medium deficient in arginine and isoleucine. For the remainder of the time, the cells were incubated at 37°C.

Ultraviolet Irradiation and Repair

The medium was decanted from the flasks or Petri dishes and saved just prior to ultraviolet irradiation. The tops were removed from the flasks or dishes and the cells were exposed to 254 nm ultraviolet radiation, delivered from one germicidal lamp (General Electric, G15 T8) mounted in a sterile transfer hood. The incident dose rate, measured with a short wave Blak-Ray meter (Ultraviolet Products, San Gabriel, Calif.) was approximately 1.0 W/m^2. Since the cells were exposed to the ultraviolet radiation for 15 seconds, the ultraviolet dose was 15 J/m^2. While one of the plates was being irradiated, the remainder of the plates were kept on ice (4°C).

[Me-^{3}H]-Thymidine (^{3}H-TdR; New England Nuclear, Boston, Mass.; 40 Ci/mmol) was added to the spent, decanted medium at 5 μCi/ml medium. This radioactive medium was then added to the cells immediately after ultraviolet irradiation. The cells were allowed to incorporate ^{3}H-Thymidine from this medium for at least 2 hours at 37°C. In one experiment cells were incubated in this medium for 2 hours at 41°C.

For long periods of ^{3}H-Thymidine incorporation, hydroxyurea was added every 24 hours to a concentration of 5 mM.

After this post-treatment incubation period, the medium was decanted and the cells were washed twice with cold phosphate buffered saline. Their detachment from the plates was facilitated by a rubber policeman in phosphate buffered saline. The detached cells were collected by centrifugation with the phosphate buffered saline decanted.

Analysis of "Unscheduled DNA Synthesis"

The pellets were usually frozen at -20°C. After thawing, the pellets were resuspended in cold 10% trichloroacetic acid (TCA). After centrifugation, the tubes were inverted to drain off the TCA. At this point the pellets were dissolved in 0.3 N KOH and incubated for 1 hour at 37°C to hydrolyse the RNA[93]. After incubation, the KOH solution was cooled and a solution was added containing enough HCl to neutralize the KOH, with TCA to be at a final concentration of 5%. Following centrifugation, the tubes were inverted to drain. The pellets were next resuspended to cold 5% TCA. The tubes were then centrifuged and inverted to drain. The tubes were again resuspended in 5% TCA and incubated for 20 minutes at 90°C to extract the DNA[94,95].

DNA was quantitated in a diphenylamine reagent[96] containing 2 g diphenylamine, 5.9 ml 61% perchloric acid, and 0.5 ml acetaldehyde (16 mg/ml) in 100 ml acetic acid. This solution was incubated for 18 hours at room temperature and compared to a highly polymerized DNA standard (Sigma Chemical Co., St. Louis, Mo.) with a Gilford spectrometer. Radioactivity in the DNA extracts was measured in a scintillation fluid containing 21 g PPO, 1.14 g dimethyl POPOP, and

150 g naphthalene per 3 liters of p-dioxane. A Packard Tri-Carb liquid scintillation spectrometer (Model 3320) was used with discriminators set between 50 and 1000 divisions and gain at 52.0%. The counting efficiency for tritium was 51.5% measured with an internal standard.

RESULTS

1. The effect of 2 hours of different pre-UV incubation temperatures on "unscheduled DNA synthesis" in V-79 cells.

Investigations were conducted on Chinese hamster lung fibroblasts (V-79) to determine if 2 hours of pre-ultraviolet (UV) hyperthermia and hypothermia affected subsequent "unscheduled DNA synthesis". The V-79 cells were inoculated into 25 cm^2 plastic flasks and were allowed to grow to confluence for 1 day at 37°C. After a 3 day incubation period at 37°C in a medium deficient in arginine and isoleucine, hydroxyurea was added to each flask to a concentration of 5 mM. The flasks were tightly sealed and were exposed for 2 hours to various temperatures before being ultraviolet irradiated at 15 J/m^2. Water baths set at 37°C, 40°C, and 43°C each contained 4 flasks, while 3 flasks were placed on ice (4°C). After the 2 hour temperature treatment, 2 flasks from each of the thermal treatment groups were UV irradiated, while the remaining flasks were not subjected to ultraviolet irradiation. After this point, all of the flasks were allowed to incorporate ^{3}H-Thymidine for 2 hours at 37°C.

The specific activity indicating the amount of "unscheduled DNA synthesis" and the amount of DNA on each plate are given in Table 1. Only 0 to 32 µg of DNA were isolated in flasks that were

Table 1. The effect of 2 hours of various temperature treatments before ultraviolet irradiation on ^{3}H-Thymidine incorporation into the DNA of Chinese hamster lung fibroblasts

MEASUREMENT	UV DOSE	TEMPERATURE BEFORE INCORPORATION			
		43°C	40°C	37°C	4°C
Weight of DNA isolated (µg/flask)	15 J/m^2	32	202	212	158
		0	172	215	105
	none	10	175	198	215
		0	200	232	
Specific activity (dpm/µg DNA)	15 J/m^2	3	170	168	124
		-	149	176	99
	none	10	68	48	56
		-	56	68	
Average specific activity difference		-	98	114	56
Average specific activity ratio		0.3	2.6	3.0	2.0

incubated at 43°C for 2 hours, while the other flasks contained roughly 200 µg of DNA, ranging from 105 tc 232 µg DNA.

Cells that were treated for 2 hours at 4°C, 37°C, and 40°C, but were not subjected to ultraviolet irradiation incorporated ^{3}H-Thymidine for 2 hours at a specific activity of roughly 60 dpm/µg DNA, ranging from 48 to 68 dpm/µg DNA. The difference between the average specific activity of cells that were ultraviolet irradiated and the average background specific activity of control cells not exposed to ultraviolet irradiation yields the specific activity attributed only to excision repair of ultraviolet induced pyrimidine dimers. Fcr cells treated in 40°C water bath, 98 dpm/µg DNA of ^{3}H-Thymidine was incorporated for excision repair and the ultraviolet irradiated cells had 2.6 times more ^{3}H-Thymidine incorporation than non-irradiated cells. For cells treated for 2 hours in a 37°C water bath, 114 dpm/µg DNA of ^{3}H-Thymidine was incorporated for excision repair and the irradiated cells had 3.0 times more ^{3}H-Thymidine incorporated than non-irradiated cells. For cells kept 2 hours on ice, 56 dpm/µg DNA of ^{3}H-Thymidine was incorporated for excision repair and the irradiated cells had twice as much ^{3}H-Thymidine incorporation as non-irradiated cells.

The results do not show a significant effect on the amount of post-UV "unscheduled DNA synthesis" caused by a 2 hour period of pre-UV hyperthermia. The 2 hour pre-UV treatment on ice appears to reduce the amount of "unscheduled DNA synthesis". Pre-UV hyperthermia at 43°C for 2 hours appears to cause significant cell detachment.

2. The effect of 8 and 21 hours of 41°C pre-UV incubation on "unscheduled DNA synthesis" in V-79 cells.

Since no effect on "unscheduled DNA synthesis" was observed with a 2 hour treatment at 40°C before UV irradiation, this period of hyperthermia was extended, with the temperature raised to 41°C, to determine if this harsher treatment would have an effect.

Chinese hamster lung fibroblasts (V-79) were inoculated into 60 mm plastic Petri dishes and were allowed to grow to confluence for 3 to 4 days at 37°C. The dishes were then incubated for 3 days in a medium deficient in arginine and isoleucine. Some of the dishes were incubated at 41°C for the final 8 hours of this 3 day period, while others were incubated at 41°C for the final 21 hours of this 3 day period. During the remainder of the 3 day period, the dishes were incubated at 37°C. Some of the other dishes were incubated only at 37°C throughout the entire 3 day period. After the 3 day period, hydroxyurea was added to a concentration of 5 mM and all the dishes were incubated for 1 hour at 37°C. At this point, some of the cells were exposed to 15 J/m^2 of ultraviolet irradiation. Finally all of the cells were allowed to incorporate ^{3}H-Thymidine for 2 hours at 37°C.

The average amount of DNA contained in the dishes was 280 µg. There was no significant difference in the amount of DNA isolated from dishes receiving different treatments. Figure 2 shows the amount of ^{3}H-Thymidine incorporation for cells that were allowed to grow for 3 days before being maintained in a medium deficient in arginine and isoleucine. For cells that were incubated at 37°C throughout the entire 3 day period in deficient medium, an average of 85 dpm/µg DNA of ^{3}H-Thymidine was incorporated for ultraviolet irradiated cells

Figure 2. The amount of ^{3}H-Thymidine (5 µCi/ml medium) incorporated into the DNA of V-79 cells for 2 hours at 37°C as a function of the previous duration of incubation at 41°C. ^{3}H-Thymidine incorporation followed the 41°C incubation period, hydroxyurea (5 mM) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2} (hollow circles) or non-irradiation (solid circles)

and 40 dpm/μg DNA for non-irradiated cells. This means that 2.1 times as much ^{3}H-Thymidine was incorporated into the DNA of irradiated cells than was incorporated into the DNA of non-irradiated cells and 45 dpm/μg DNA of ^{3}H-Thymidine was incorporated in the excision repair of ultraviolet radiation induced dimers. For cells that were incubated at 41°C for the final 8 hours in deficient medium before hydroxyurea treatment, an average of 104 dpm/μg DNA of ^{3}H-Thymidine incorporation was observed in UV irradiated cells, while an average of 38 dpm/μg DNA was observed in non-irradiated cells. Thus, irradiated cells incorporated 2.7 times as much ^{3}H-Thymidine as non-irradiated ones and 66 dpm/μg DNA was incorporated during excision repair. For cells incubated at 41°C for 21 hours before hydroxyurea treatment, an average of 72 dpm/μg DNA of ^{3}H-Thymidine was observed in irradiated cells, while 29 dpm/μg DNA was observed in non-irradiated cells. Irradiated cells incorporated 2.5 times as much radioactivity as non-irradiated cells, and 43 dpm/μg DNA was incorporated in excision repair.

Figure 3 shows the amount of "unscheduled DNA synthesis" in cells that were allowed to grow for 4 days before being maintained in the deficient medium. For cells that were only incubated at 37°C for the 3 day period in deficient medium, an average of 116 dpm/μg DNA of ^{3}H-Thymidine was incorporated in ultraviolet irradiated cells and an average of 41 dpm/μg DNA in non-irradiated cells. Irradiated cells incorporated 2.8 times as much ^{3}H-Thymidine as non-irradiated cells, and 75 dpm/μg DNA was incorporated in excision repair. Irradiated cells that were incubated at 41°C for 8 hours before hydroxyurea treatment incorporated, on the average, 101 dpm/μg DNA of ^{3}H-Thymidine. For cells that were incubated at 41°C for 21 hours before hydroxyurea

Figure 3. The amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells for 2 hours at 37°C as a function of the previous duration of incubation at 41°C. ^{3}H-Thymidine incorporation followed the 41°C incubation period, hydroxyurea (5 mM) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2} (hollow circles) or non-irradiation (solid circles)

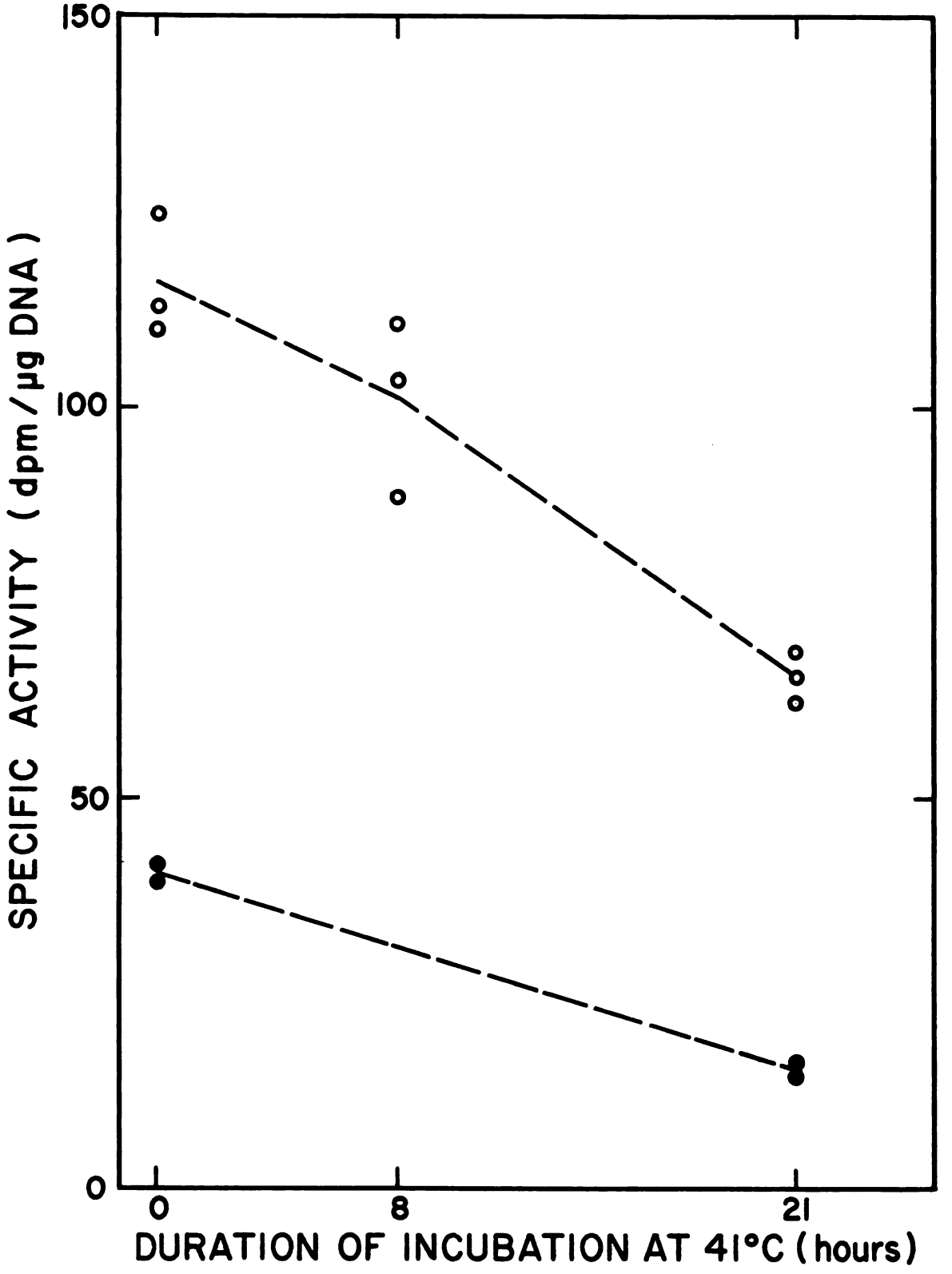

150
100
50
0
SPECIFIC ACTIVITY (dpm / µg DNA)
0
8
21
DURATION OF INCUBATION AT 41°C (hours)

Figure 3.

treatment, an average of 66 dpm/μg DNA of ^{3}H-Thymidine was incorporated in irradiated cells and an average of 16 dpm/μg DNA in non-irradiated cells. Irradiated cells incorporated 4.1 times as much ^{3}H-Thymidine as non-irradiated cells and 50 dpm/μg DNA was used in excision repair.

The results show that a 21 hour period at 41°C before UV irradiation appears to reduce the amount of "unscheduled DNA synthesis" after UV irradiation. However there appears to be some variation in the amount of "unscheduled DNA synthesis" between the two experiments depicted in Figures 2 and 3 for cells that were incubated at 41°C for 0 and 8 hours before hydroxyurea treatment. This variation might be dependent on when the Petri dishes were initially seeded and growth time before being maintained in deficient medium.

3. The effect of 3 days of 41°C pre-UV incubation on "unscheduled DNA synthesis" in V-79 cells.

The period of pre-UV hyperthermia was extended to 3 days to determine if this would cause any further reduction in "unscheduled DNA synthesis" after UV irradiation. Furthermore, some of the cells were exposed to a higher temperature during the period of pre-UV treatment with hydroxyurea, while other cells were, in addition, incubated at the raised temperature during ^{3}H-Thymidine incorporation to determine if these treatments affected the amounts of "unscheduled DNA synthesis".

Chinese hamster lung fibroblasts were inoculated into 60 mm plastic Petri dishes and were grown to confluence at 37°C for 2 to 3 days. At confluence, they were maintained in a medium deficient in arginine and isoleucine for 3 days either at 37°C or at 41°C. At this point hydroxyurea was added and the cells were incubated for

1 hour at either 37°C or 41°C. After some of the plates were ultraviolet irradiated, all of the cells were allowed to incorporate ^{3}H-Thymidine for 2 hours at 37°C or at 41°C.

The amount of "unscheduled DNA synthesis" in cells which took 3 days to reach confluence is shown in Figure 4. All of these plates were incubated with hydroxyurea at 37°C for 1 hour and allowed to incorporate ^{3}H-Thymidine for 2 hours at 37°C. The amount of DNA isolated ranged from 176 µg to 188 µg, averaging 185 µg, from cells that were always incubated at 37°C. Cells treated with ultraviolet radiation incorporated, on the average, 194 dpm/µg DNA of ^{3}H-Thymidine, while non-irradiated cells incorporated 40 dpm/µg DNA. Irradiated cells incorporated 4.9 times as much ^{3}H-Thymidine as non-irradiated cells and 154 dpm/µg DNA was used in excision repair.

The amount of DNA isolated from cells incubated at 41°C for 3 days in deficient medium ranged from 116 µg to 151 µg, averaging 134 µg. On the average, irradiated cells incorporated 95 dpm/µg DNA, while non-irradiated cells incorporated 16 dpm/µg DNA. Irradiated cells incorporated 6.0 times as much ^{3}H-Thymidine as non-irradiated cells and 79 dpm/µg DNA was used in excision repair.

Figure 5 shows the amount of "unscheduled DNA synthesis" in cells which took 2 days to reach confluence. For cells which were incubated in deficient medium for 3 days ("pre-HU") at 37°C, incubated with hydroxyurea ("HU") at 37°C for 1 hour, and allowed to incorporate ^{3}H-Thymidine ("repaired") at 37°C for 2 hours, 194 to 251 µg of DNA was isolated per plate, averaging 236 µg. Irradiated cells averaged 107 dpm/µg DNA of ^{3}H-Thymidine incorporation, while non-irradiated cells incorporated 24 dpm/µg DNA. Irradiated cells incorporated

Figure 4. The amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells for 2 hours at 37°C as a function of the previous duration of incubation at 41°C. ^{3}H-Thymidine incorporation followed the 41°C incubation period, hydroxyurea (5 mM) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2} (hollow circles) or non-irradiation (solid circles)

Figure 5. The amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells for 2 hours at 37°C following 254 nm ultraviolet irradiation at 15 J/m^{2} (hollow circles) or non-irradiation (solid circles) as a function of the temperatures of (a) the 72 hour incubation in deficient medium before hydroxyurea was added ("pre-HU"), (b) the 1 hour incubation with hydroxyurea (5 mM) before ^{3}H-Thymidine incorporation ("HU"), and (c) the 2 hour ^{3}H-Thymidine incorporation ("repair")

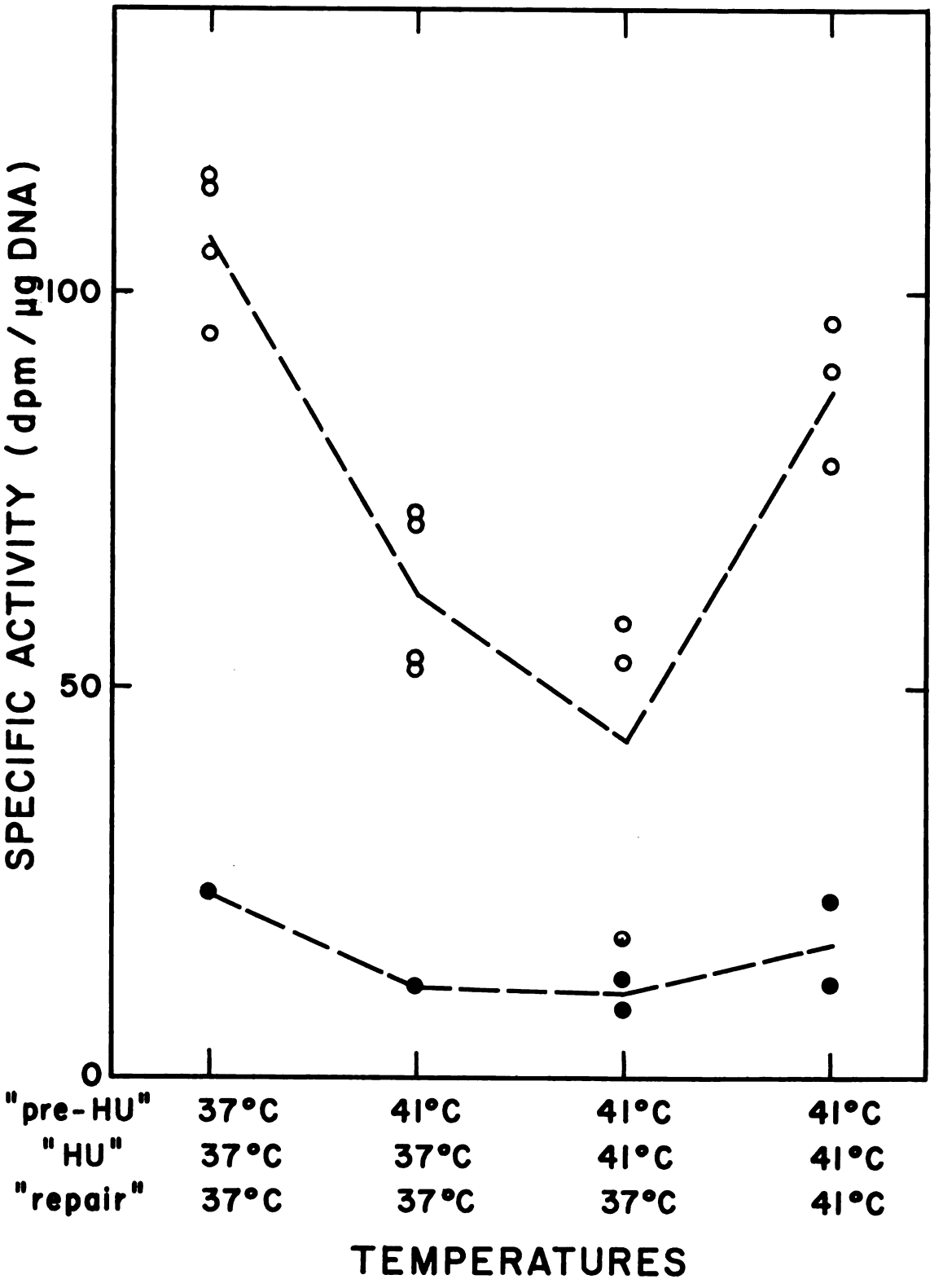

SPECIFIC ACTIVITY (dpm / µg DNA)
100
50
0
"pre-HU" 37°C 41°C 41°C 41°C
"HU" 37°C 37°C 41°C 41°C
"repair" 37°C 37°C 37°C 41°C
TEMPERATURES

Figure 5.

4.5 times more than non-irradiated ones and 83 dpm/μg DNA was due to excision repair.

For cells "pre-HU" incubated at 41°C, "HU" incubated at 37°C, and "repaired" at 37°C, DNA was isolated at 111 to 173 μg per plate, averaging 153 μg. Irradiated cells averaged 62 dpm/μg DNA incorporation, while non-irradiated cells incorporated 12 dpm/μg DNA. Irradiated cells incorporated 5.2 times more than non-irradiated ones and 50 dpm/μg DNA was due to excision repair.

For cells "pre-HU" incubated at 41°C, "HU" incubated at 41°C, and "repaired" at 37°C, DNA was isolated at 134 to 189 μg per plate, averaging 162 μg. Irradiated cells averaged 43 dpm/μg DNA incorporation, while non-irradiated cells averaged 11 dpm/μg DNA. Irradiated cells incorporated 3.9 times more than non-irradiated ones and 32 dpm/μg DNA was due to excision repair.

For cells "pre-HU" incubated at 41°C, "HU" incubated at 41°C, and "repaired" at 41°C, DNA was isolated at 90 to 129 μg per plate, averaging 104 μg. Irradiated cells averaged 88 dpm/μg DNA incorporation, while non-irradiated ones averaged 18 dpm/μg DNA. Irradiated cells incorporated 4.9 times more than non-irradiated ones and 70 dpm/μg DNA was due to excision repair.

Therefore a 3 day period at 41°C before UV irradiation significantly reduced the amount of "unscheduled DNA synthesis". Also more ^{3}H-Thymidine was incorporated in a 2 hour post-UV period at 41°C than at 37°C when the cells were incubated at 41°C for 3 days before ultraviolet irradiation. The amount of "unscheduled DNA synthesis" either decreased or remained at approximately the same level when the temperature of the pre-UV incubation with

hydroxyurea was raised from 37°C to 41°C. Non-irradiated controls measuring residual "semi-conservative DNA synthesis" did not demonstrate any significant change in specific activity with the temperature raised in the one hour pre-UV incubation with hydroxyurea. This indicates that the hyperthermia did not break down a sufficient amount of hydroxyurea that would result in an increase in "semi-conservative DNA synthesis".

4. The effect of cycloheximide and 3 days of 41°C pre-UV incubation on "unscheduled DNA synthesis" in V-79 cells.

Cycloheximide, an inhibitor of protein synthesis, was added to V-79 cells one hour before UV irradiation to determine if this treatment would further reduce the amount of "unscheduled DNA synthesis". This would be expected if the cells that did not receive cycloheximide were synthesizing additional DNA excision repair proteins during UV irradiation and ^{3}H-Thymidine incorporation.

Chinese hamster lung fibroblasts were allowed to grow to confluence at 37°C for 2 days. The cells were incubated at 37°C or at 41°C for 3 days in a medium deficient in arginine and isoleucine. At this point hydroxyurea was added to each of the dishes at a concentration of 5 mM and cycloheximide was added to some of the dishes at a concentration of 5 µg/ml medium. The cells were incubated for one hour at 37°C. After some of the cells were UV irradiated at 15 J/m^{2}, all of the cells were allowed to incorporate ^{3}H-Thymidine for 2 hours at 37°C in the spent medium containing hydroxyurea and sometimes cycloheximide. The results are shown in Figure 6.

Cells incubated for 3 days in deficient medium at 41°C and then treated with cycloheximide contained 50 to 84 µg DNA per plate,

Figure 6. The amount of ^{3}H-Thymidine (5 µCi/ml medium) incorporated into the DNA of V-79 cells for 2 hours at 37°C as a function of previous durations of incubation at 41°C. ^{3}H-Thymidine incorporation followed the 41°C incubation period, treatment for 1 hour with hydroxyurea (5 mM) and with (hollow symbols) or without (solid symbols) cycloheximide (5 µg/ml), and 254 nm ultraviolet irradiation at 15 J/m^{2} (squares) or non-irradiation (circles)

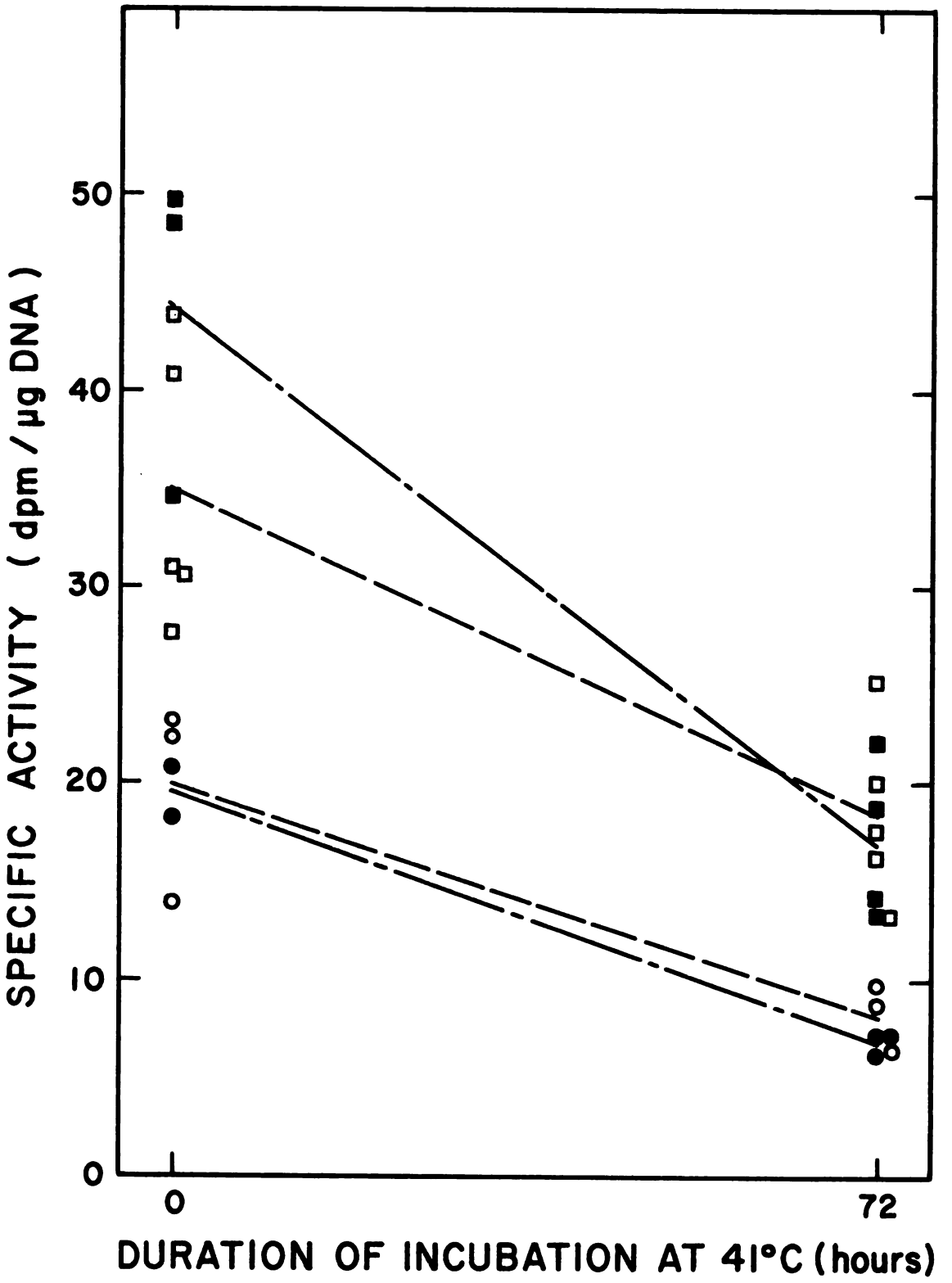

SPECIFIC ACTIVITY (dpm / µg DNA)
50
40
30
20
10
0
0
72
DURATION OF INCUBATION AT 41°C (hours)

Figure 6.

averaging 65 µg. Irradiated cell ^{3}H-Thymidine incorporation averaged 18.5 dpm/µg DNA, while non-irradiated cells averaged 8.4 dpm/µg DNA. Irradiated cells incorporated 2.2 times more than non-irradiated cells and 10.1 dpm/µg DNA was due to excision repair.

Cells incubated for 3 days in deficient medium at 41°C, but not treated with cycloheximide, contained 50 to 90 µg DNA per plate, averaging 72 µg. Irradiated cells averaged 17.2 dpm/µg DNA incorporation, while non-irradiated cells averaged 7.0 dpm/µg DNA. Irradiated cells incorporated 2.5 times more than non-irradiated cells and 10.2 dpm/µg DNA was due to excision repair.

Cells that were always incubated at 37°C and treated with cycloheximide contained 66 to 91 µg DNA per plate, averaging 71 µg. Irradiated cells averaged 34.8 dpm/µg DNA incorporation, while non-irradiated cells averaged 19.8 dpm/µg DNA. Irradiated cells incorporated 1.8 times more than non-irradiated cells and 15.0 dpm/µg DNA was due to excision repair.

Cells always incubated at 37°C and not treated with cycloheximide contained 70 to 91 µg DNA per plate, averaging 84 µg. Irradiated cells averaged 44.2 dpm/µg DNA incorporation, while non-irradiated cells averaged 19.4 dpm/µg DNA. Irradiated cells incorporated 2.3 times more than non-irradiated ones, and 24.8 dpm/µg DNA was due to excision repair.

The results show that cycloheximide did not alter the amount of "unscheduled DNA synthesis" in cells pre-UV incubated for 3 days at either 37°C or at 41°C.

5. The effect of incubation at 41°C for various spans of time before ultraviolet irradiation on "unscheduled DNA synthesis" in V-79 cells.

An experiment was conducted to determine if the amount of "unscheduled DNA synthesis" decreased regularly with increasing time of pre-UV hyperthermia.

Chinese hamster lung fibroblasts were inoculated into 60 mm plastic Petri dishes and were grown to confluence at 37°C. At confluence the cells were maintained for 72 hours in a medium deficient in arginine and isoleucine. The dishes were incubated at 37°C for the first 0, 12, 24, 36, 48, 60, and 72 hours of this 72 hour period. The cells were then incubated at 41°C for the remaining time of the 72 hour period. After this 3 day period, the cells were treated with hydroxyurea and incubated at 37°C for 1 hour. At this point some of the plates were exposed to 15 J/m^2 of ultraviolet irradiation and all of the cells were allowed to incorporate ^{3}H-Thymidine at 37°C for 2 hours. The results are shown in Figure 7.

As shown in the top of the Figure, the DNA isolated was less than 100 µg per plate and usually greater than 50 µg per plate. There is a slight tendency for the amount of DNA isolated to decrease with greater amounts of time the cells were incubated at 41°C. There does not appear to be a pattern with the amount of DNA isolated and whether or not the cells were exposed to ultraviolet irradiation.

There also is a slight tendency for less ^{3}H-Thymidine incorporation in non-irradiated cells with greater periods of incubation at 41°C. The amount of incorporation decreased from an average of 54 dpm/µg DNA for non-irradiated cells incubated always at 37°C to

Figure 7. The amount of DNA isolated (top) and ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA (bottom) of V-79 cells for 2 hours at 37°C as a function of the previous duration of incubation at 41°C. ^{3}H-Thymidine incorporation followed the 41°C incubation period, hydroxyurea (5 m<u>M</u>) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2} (hollow circles) or non-irradiation (solid circles)

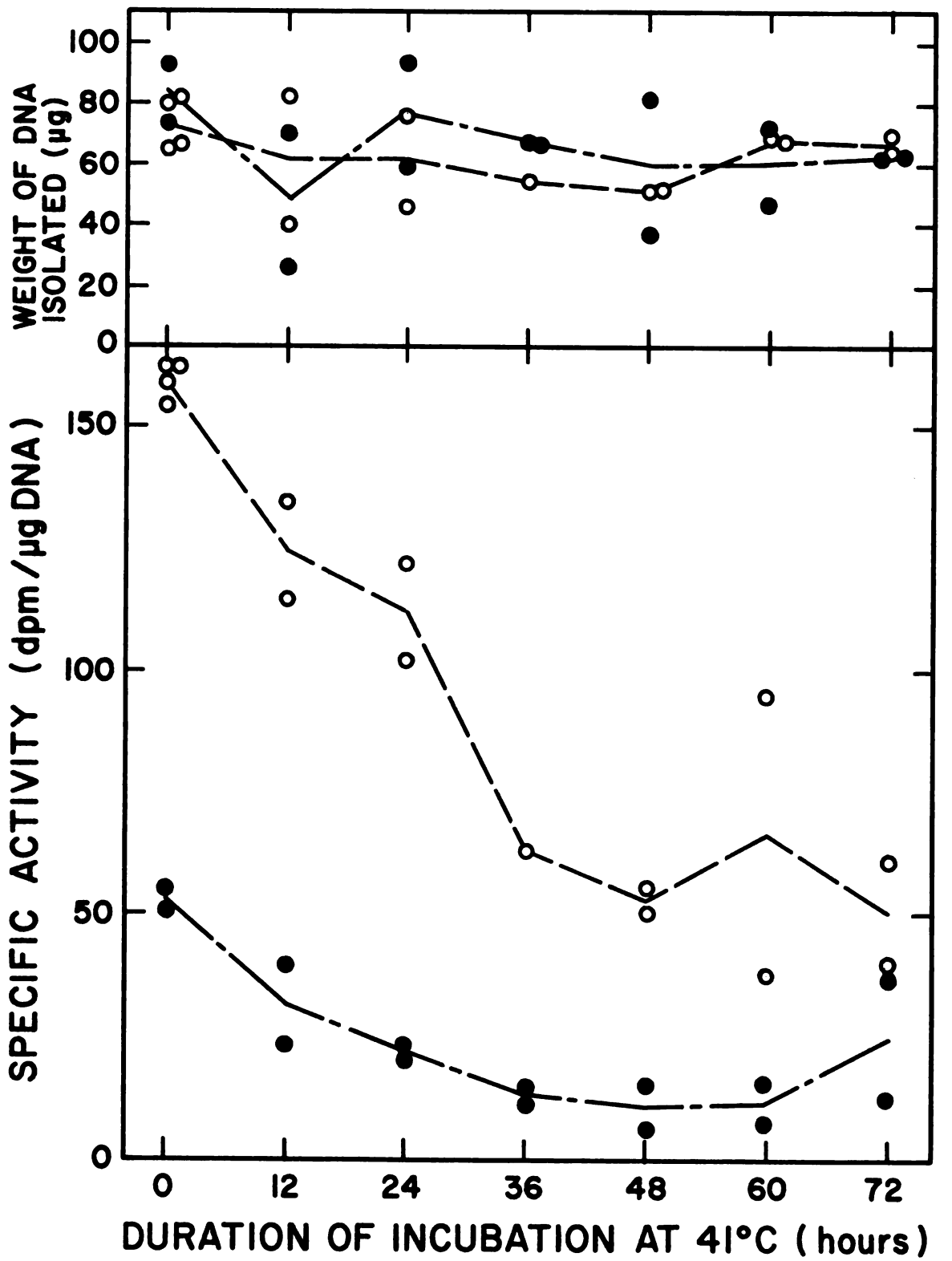

WEIGHT OF DNA ISOLATED (µg)
100
80
60
40
20
0
SPECIFIC ACTIVITY (dpm/µg DNA)
150
100
50
0
0
12
24
36
48
60
72
DURATION OF INCUBATION AT 41°C (hours)

Figure 7.

an average of 25 dpm/μg DNA for cells incubated at 41°C for 3 days.

Cells that were exposed to ultraviolet radiation showed a much greater decrease in ^{3}H-Thymidine incorporation with greater periods of incubation at 41°C. Incorporation decreased from 162 dpm/μg DNA for irradiated cells always incubated at 37°C to 50 dpm/μg DNA for irradiated cells that were previously incubated at 41°C for 3 days. For cells never incubated at 41°C, irradiated cells incorporated 3.0 times more ^{3}H-Thymidine than non-irradiated cells, while for cells incubated at 41°C for 3 days, irradiated cells incorporated 2.0 times more than non-irradiated cells.

Figure 8 shows the amount of ^{3}H-Thymidine incorporated in excision repair as a function of the time exposed at 41°C immediately prior to UV irradiation. Each point represents the difference between the specific activity of the average amount of ^{3}H-Thymidine incorporated in ultraviolet irradiated cells and the average specific activity of the non-irradiated cells, resulting in the net specific activity attributed only to the excision repair of ultraviolet induced dimers. The results indicate that the net specific activity decreases from a value of 108 dpm/μg DNA for cells only incubated at 37°C to a value of 25 dpm/μg DNA for cells incubated at 41°C for three days immediately prior to ultraviolet irradiation.

6. <u>The effect of incubation at 33°C for various spans of time before ultraviolet irradiation on ^{3}H-Thymidine incorporation in V-79 cells</u>.

Since pre-UV hyperthermia appears to decrease the amount of subsequent ^{3}H-Thymidine incorporation, experiments were conducted to

Figure 8. The net amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells for 2 hours at 37°C, which was used for the excision repair of ultraviolet radiation-induced dimers, as a function of previous durations of incubation at 41°C. Each point is derived from the difference of the averaged values of points in Figure 7 between those representing ultraviolet irradiated cells and non-irradiated cells

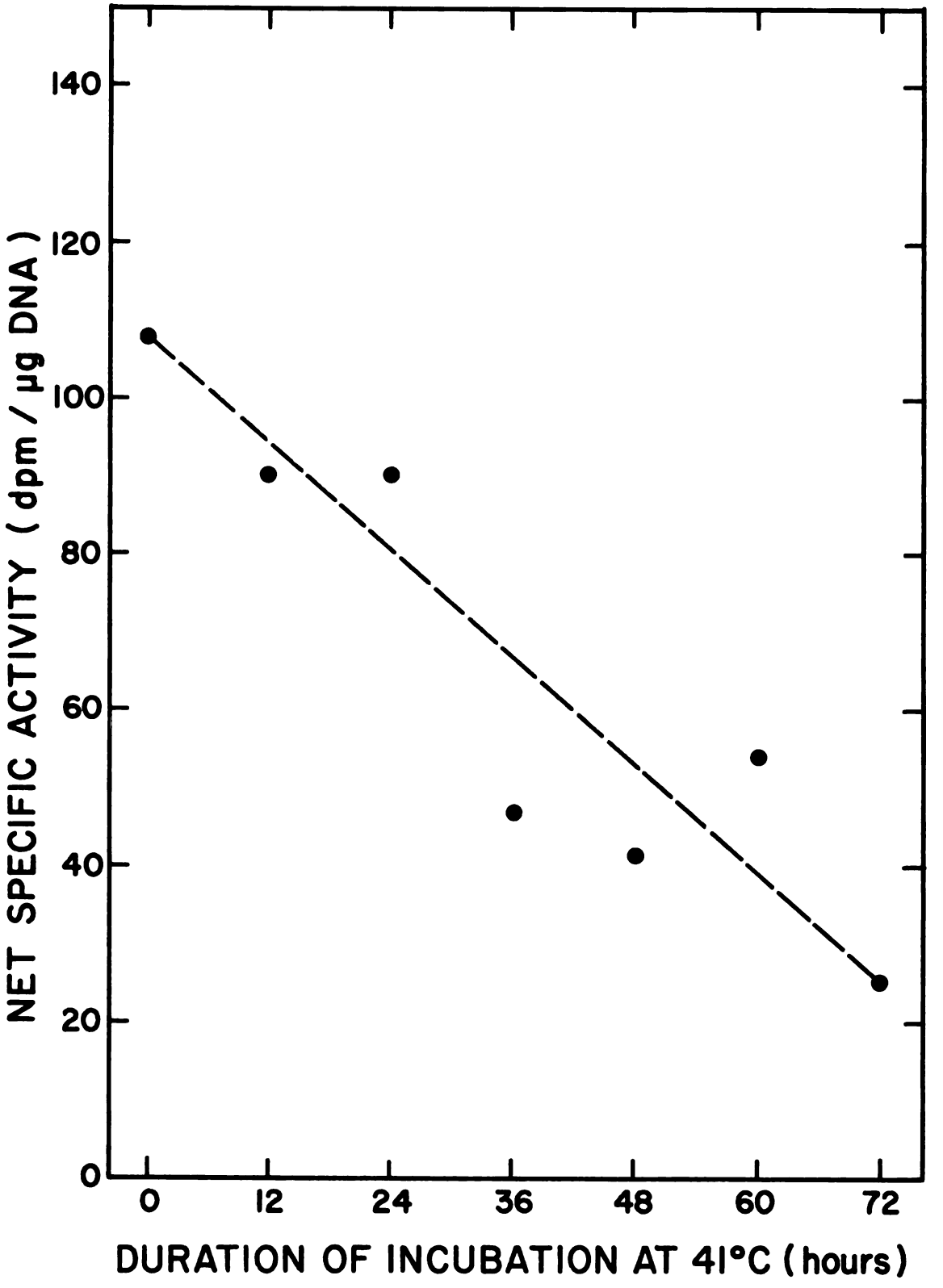

NET SPECIFIC ACTIVITY (dpm / µg DNA)
140
120
100
80
60
40
20
0
0
12
24
36
48
60
72
DURATION OF INCUBATION AT 41°C (hours)

Figure 8.

determine if pre-UV hypothermia also affects the amount of ^{3}H-Thymidine incorporation.

Chinese hamster lung fibroblasts were inoculated into 60 mm plastic Petri dishes and were allowed to grow to confluence for 24 hours at 33°C and 37°C. At confluence the cells were maintained for 72 hours in a medium deficient in arginine and isoleucine. The cells which were grown at 33°C were incubated at 33°C throughout the entire 72 hour period. The cells which were grown at 37°C were incubated at 37°C for the first 0, 24, 48, and 72 hours and incubated at 33°C for the remaining time of the 72 hour period in the deficient medium. In the final 1 hour of the 72 hour period, the cells were incubated with hydroxyurea at the same temperature at which they were incubated for the past 23 hours. All of the cells were then exposed to 15 J/m^{2} of ultraviolet radiation and then allowed to incorporate ^{3}H-Thymidine for 2 hours at 37°C.

The results for one of these experiments are shown in Figure 9. The amount of DNA isolated in this experiment averaged 403 µg, ranging from 347 to 447 µg DNA per plate. The amount of ^{3}H-Thymidine incorporation was 22.1 dpm/µg DNA for cells incubated at 37°C for 4 days and was 7.2 dpm/µg DNA for cells incubated at 33°C for 4 days before ultraviolet irradiation.

The results for a repeated experiment are shown in Figure 10. The amount of DNA isolated in this experiment averaged 360 µg per plate, ranging from 265 µg to 451 µg per plate. The amount of ^{3}H-Thymidine incorporation decreased from a value of 41.0 dpm/µg DNA for cells incubated at 37°C for 4 days before ultraviolet

Figure 9. The amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells for 2 hours at 37°C as a function of previous durations of incubation at 33°C. ^{3}H-Thymidine incorporation followed the 33°C incubation period, hydroxyurea (5 mM) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2}

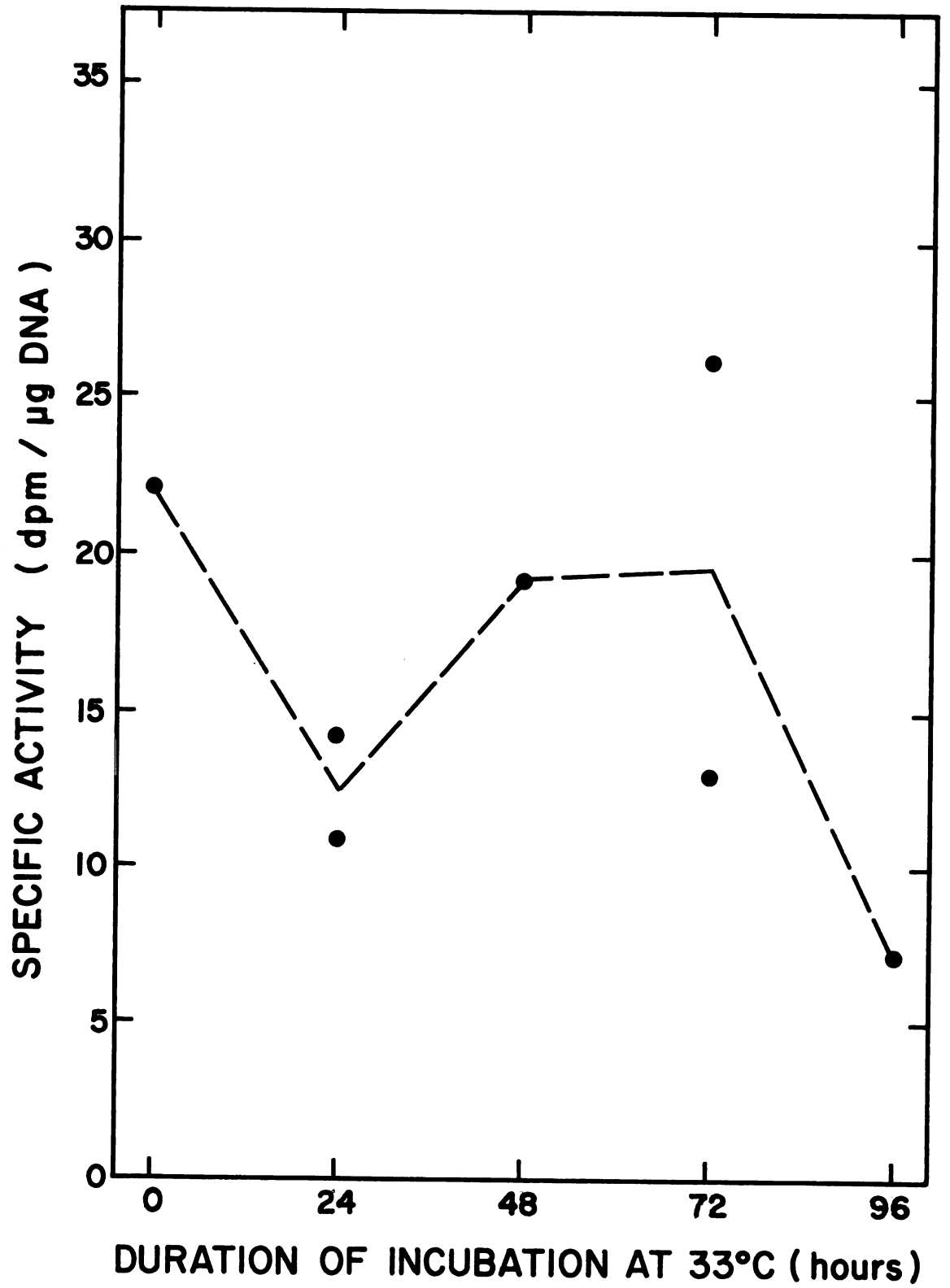

SPECIFIC ACTIVITY (dpm / µg DNA)
35
30
25
20
15
10
5
0
0
24
48
72
96
DURATION OF INCUBATION AT 33°C (hours)

Figure 9.

Figure 10. The amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells for 2 hours at 37°C as a function of previous durations of incubation at 33°C. ^{3}H-Thymidine incorporation followed the 33°C incubation period, hydroxyurea (5 mM) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2}

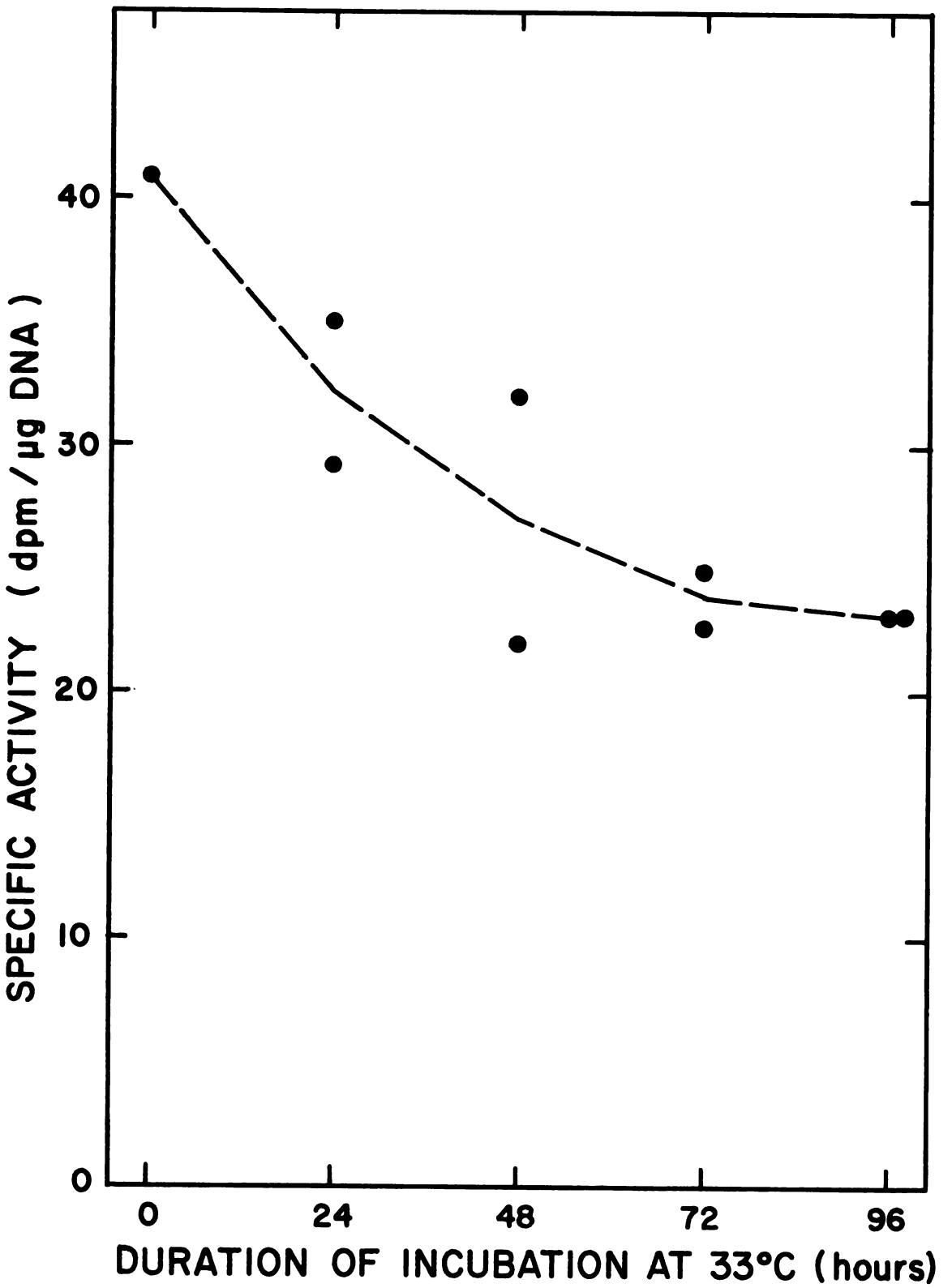

SPECIFIC ACTIVITY (dpm / µg DNA)
0
10
20
30
40
0
24
48
72
96
DURATION OF INCUBATION AT 33°C (hours)

Figure 10.

irradiation to a value of 23.2 dpm/μg DNA for cells incubated at 33°C for 4 days before ultraviolet irradiation.

While not conclusive, the results from these two experiments suggest that a period of pre-UV hypothermia might decrease the amount of ^{3}H-Thymidine incorporation.

7. The effect of incubation at 33°C for various spans of time before ultraviolet irradiation on various periods of ^{3}H-Thymidine incorporation in V-79 cells.

An experiment was conducted to determine the effect of pre-UV hypothermia on the amount of ^{3}H-Thymidine incorporation for extended lengths of time after UV irradiation. The procedure was identical to the previous two experiments in Section 6, except that ^{3}H-Thymidine was incorporated at 37°C for 5, 10, and 20 hours after ultraviolet irradiation, rather than for 2 hours. The results are shown in Figure 11.

Cells allowed to incorporate ^{3}H-Thymidine for 5 hours had an average specific activity of 197 dpm/μg DNA, ranging from 144 to 375 dpm/μg DNA. The amount of DNA isolated from these cells averaged 66 μg per plate, ranging from 50 to 102 μg per plate.

Cells incubated for 10 hours after ultraviolet irradiation incorporated ^{3}H-Thymidine at an average of 300 dpm/μg DNA, ranging from 228 to 388 dpm/μg DNA. The amount of DNA isolated averaged 55 μg DNA per plate, ranging from 41 to 71 μg per plate.

Cells incubated for 20 hours after ultraviolet irradiation incorporated ^{3}H-Thymidine at an average of 518 dpm/μg DNA, ranging from 391 to 616 dpm/μg DNA. The amount of DNA isolated averaged 47 μg per plate, ranging from 23 to 81 μg per plate.

The results indicate that even though ^{3}H-Thymidine incorporation into DNA increased when allowed to proceed for longer periods of time,

Figure 11. The amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells for various periods at 37°C as a function of previous durations of incubation at 33°C. ^{3}H-Thymidine incorporation for 5 hours (solid circles), 10 hours (hollow circles), and 20 hours (squares) followed the 33°C incubation period, hydroxyurea (5 m$\underline{M}$) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2}

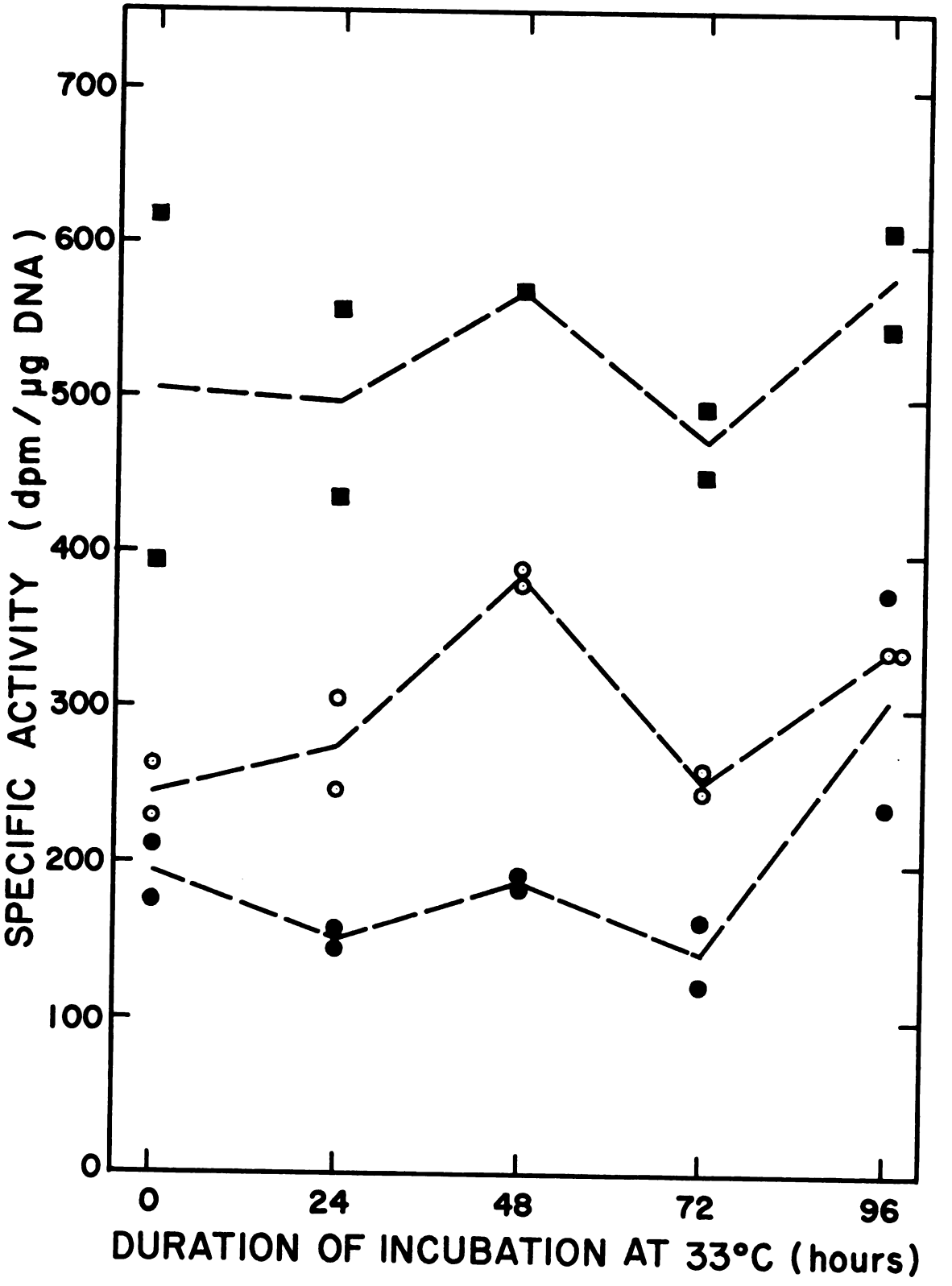

700
600
500
400
300
200
100
0
SPECIFIC ACTIVITY (dpm / µg DNA)
0
24
48
72
96
DURATION OF INCUBATION AT 33°C (hours)

Figure 11.

the pre-UV hypothermia did not appear to affect the amount of ^{3}H-Thymidine incorporation.

8. The effect of incubation at 33°C for 5 days before ultraviolet irradiation on various periods of ^{3}H-Thymidine incorporation in V-79 cells.

An experiment was conducted to determine the effect of pre-UV hypothermia for 5 days on ^{3}H-Thymidine incorporation for durations up to 68 hours in Chinese hamster lung fibroblasts. Cells were inoculated into 60 mm plastic Petri dishes, were allowed to grow to confluence for 48 hours, and then were maintained in a medium deficient in arginine and isoleucine for 72 hours. During this entire 120 hour period, the cells were either incubated at 33°C or at 37°C. Hydroxyurea was added in the final hour of this 120 hour period. At this point, all the cells were exposed to ultraviolet radiation and were allowed to incorporate ^{3}H-Thymidine at 37°C for 2, 5, 10, 20, 32, 44, 56, and 68 hours.

The results for ^{3}H-Thymidine incorporation up to 32 hours are shown in Figure 12. According to the top of the Figure, the DNA isolated decreased from an average of 32 µg per plate with 2 hours of ^{3}H-Thymidine incorporation to an average of 24 µg per plate for 32 hours of incorporation. For cells incubated for 5 days at 33°C before ultraviolet irradiation, 2 hours of ^{3}H-Thymidine incorporation resulted in a specific activity of an average of 360 dpm/µg DNA and this value increased to an average of 2218 dpm/µg DNA for 32 hours of incubation. For cells incubated for 5 days at 37°C before ultraviolet irradiation, the average specific activity increased from 112 dpm/µg DNA for 2 hours incorporation to 1920 dpm/µg DNA for 32 hours incorporation.

Figure 12. The amount of DNA isolated (top) and ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA (bottom) of V-79 cells at 37°C as a function of the duration of ^{3}H-Thymidine incorporation, following a 120 hour incubation period at either 37°C (solid circles) or 33°C (hollow circles), hydroxyurea (5 mM) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2}

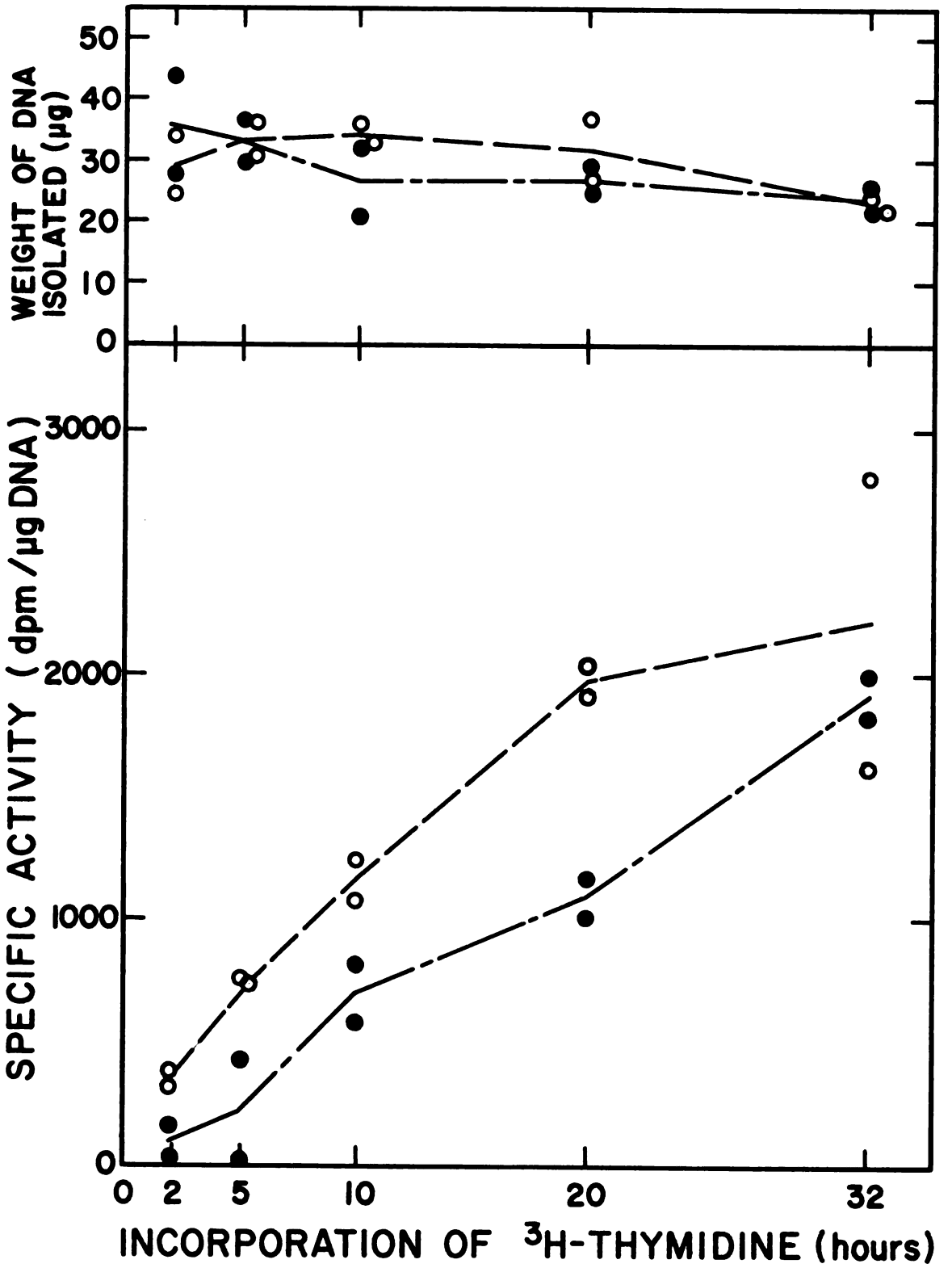

WEIGHT OF DNA ISOLATED (µg)
50
40
30
20
10
0
SPECIFIC ACTIVITY (dpm/µg DNA)
3000
2000
1000
0
0
2
5
10
20
32
INCORPORATION OF ³H-THYMIDINE (hours)

Figure 12.

The results indicate that 5 days of pre-UV hypothermia caused an increase in the amount of ^{3}H-Thymidine incorporation for periods up to 32 hours after UV irradiation. After 32 hours of incubation following UV irradiation, the amount of ^{3}H-Thymidine incorporated into DNA increased dramatically having specific activities sometimes greater than 100,000 dpm/μg DNA. This may indicate that hydroxyurea breaks down after 32 hours allowing "semi-conservative DNA synthesis" to proceed.

9. The effect of incubation at 33°C for 5 days before ultraviolet irradiation on "unscheduled DNA synthesis" conducted for various durations in V-79 cells with added hydroxyurea.

Two additional experiments were conducted to determine the effect of pre-UV hypothermia on the amount of "unscheduled DNA synthesis" after UV irradiation. Hydroxyurea was added at various points during ^{3}H-Thymidine incorporation to prevent "semi-conservative DNA synthesis" from resuming.

This experiment was identical to the one previously described in Section 8 except that ^{3}H-Thymidine was allowed to incorporate at 37°C for 2, 5, 16, 29, 41, 51, 63, 75, and 87 hours after ultraviolet irradiation. Hydroxyurea was added at 17, 43, and 68 hours after ultraviolet irradiation, as well as 1 hour before irradiation, in increasing increments of 5 mM each. Controls that were not exposed to ultraviolet irradiation were treated in an otherwise identical manner as those that were irradiated.

The results are shown in Figures 13 and 14. The top of Figure 13 shows the amount of DNA isolated from each plate. The amount of DNA isolated decreased slightly from an average of 42 μg DNA from plates allowed to incorporate ^{3}H-Thymidine for 2 hours at 37°C to an

Figure 13. The amount of DNA isolated (top) and ^{3}H-Thymidine (5 µCi/ml medium) incorporated into the DNA (bottom) of V-79 cells at 37°C as a function of the duration of ^{3}H-Thymidine incorporation, following a 120 hour incubation period at either 37°C (solid symbols) or 33°C (hollow symbols) and 254 nm ultraviolet irradiation at 15 J/m^{2} (squares) or non-irradiation (circles). Hydroxyurea ("HU") was added at 17, 43, and 68 hours after ultraviolet irradiation, as well as 1 hour before irradiation, in increasing increments of 5 m$\underline{M}$ each

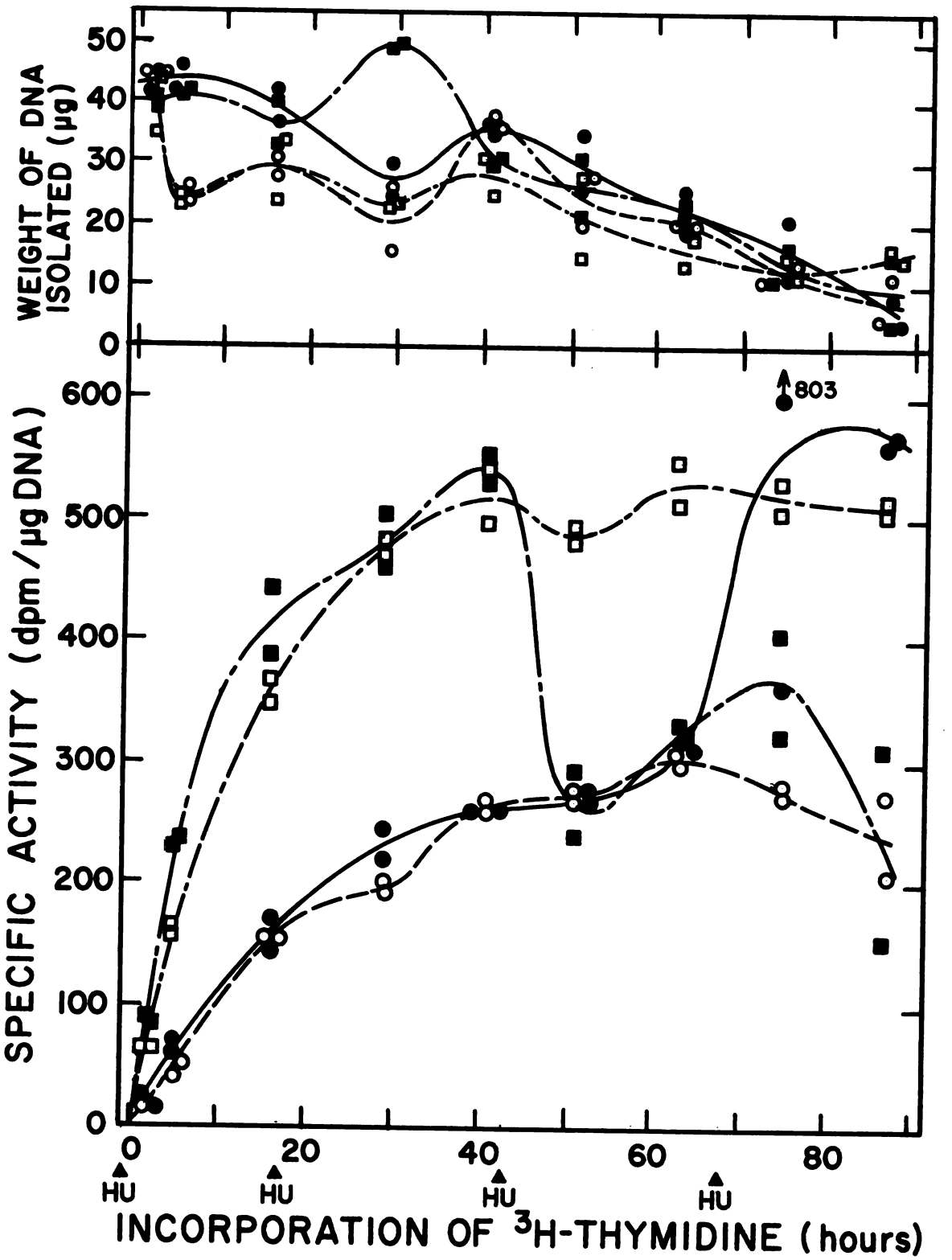

WEIGHT OF DNA ISOLATED (μg)
50
40
30
20
10
0
803
SPECIFIC ACTIVITY (dpm/μg DNA)
600
500
400
300
200
100
0
0
20
40
60
80
HU
HU
HU
HU
INCORPORATION OF ^{3}H-THYMIDINE (hours)

Figure 13.

Figure 14. The net amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells at 37°C, which was used for the excision repair of ultraviolet radiation-induced dimers, as a function of the duration of ^{3}H-Thymidine incorporation. Each point is derived from the difference of the averaged values of points in Figure 13 between those representing ultraviolet irradiated cells and non-irradiated cells, which were previously incubated for 120 hours at either 37°C (solid circles) or 33°C (hollow circles)

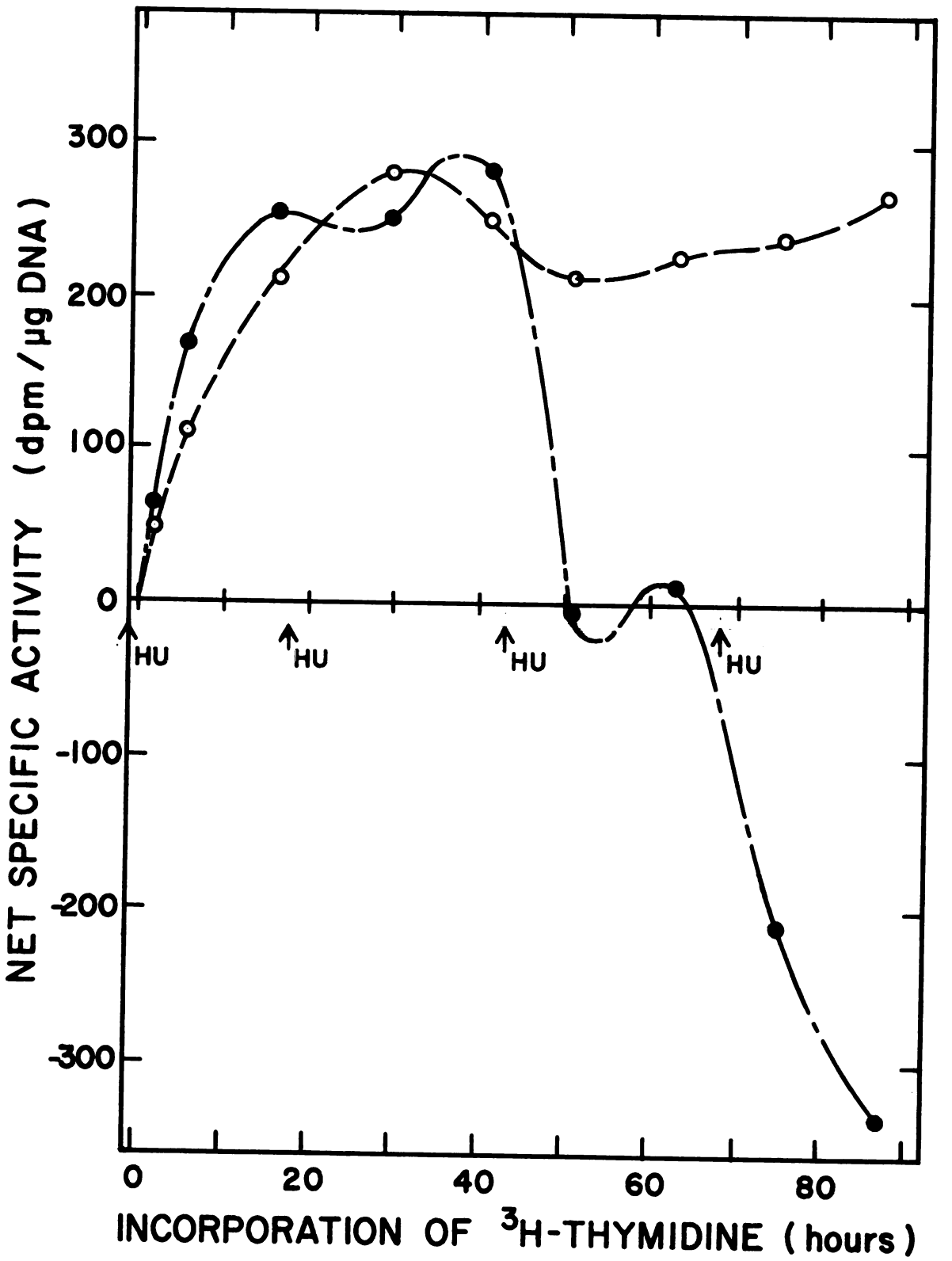

300
200
100
0
-100
-200
-300
NET SPECIFIC ACTIVITY (dpm/µg DNA)
HU
HU
HU
HU
0
20
40
60
80
INCORPORATION OF ^{3}H-THYMIDINE (hours)

Figure 14.

average value of 33 μg from plates allowed to incorporate for 41 hours, and this value decreased to an average of 10 μg from plates allowed to incorporate for 87 hours. It appeared that the treatments of a 5 day incubation at 33°C, or ultraviolet irradiation, did not affect the amount of DNA eventually isolated, while the incorporation of ^{3}H-Thymidine at 37°C resulted in decreasing amounts of DNA isolated with increasing times of incubation with the radioactive label.

The bottom of Figure 13 shows the amount of ^{3}H-Thymidine incorporation per unit weight of DNA as a function of the length of the period of incorporation. For cells incubated at 37°C for 5 days before ultraviolet irradiation, ^{3}H-Thymidine incorporation increased from an average of 84 dpm/μg DNA for 2 hours of incorporation to an average of 544 dpm/μg DNA for 41 hours of incorporation, and then decreased to an average of 232 dpm/μg DNA for 87 hours of incorporation. For cells incubated at 37°C for 5 days, but not exposed to ultraviolet irradiation, the specific activity increased from an average 20 dpm/μg DNA for 2 hours incorporation, to an average of 259 dpm/μg DNA for 41 hour incorporation, and to an average of 565 dpm/μg DNA for 87 hour incorporation. For cells incubated at 33°C for 5 days before ultraviolet irradiation, the specific activity mean values increased from 62 dpm/μg DNA for 2 hour incorporation to 518 dpm/μg DNA for 41 hour incorporation, and stayed relatively constant to 510 dpm/μg DNA for 87 hour incorporation. For cells incubated at 33°C for 5 days, but not UV irradiated, the specific activity mean values increased from 13 dpm/μg DNA for 2 hour incorporation to 266 dpm/μg DNA for 41 hour

incorporation, and stayed relatively constant to 240 dpm/μg DNA for 87 hour incorporation.

Figure 14 shows the net specific activity attributed only to excision repair of ultraviolet induced dimers as a function of the period of ^{3}H-Thymidine incorporation, which is derived from the difference of the mean specific activities between ultraviolet irradiated cells and non-irradiated cells. For cells incubated at 33°C for 5 days, this amount of "unscheduled DNA synthesis" due to ultraviolet irradiation increased from 49 dpm/μg DNA for 2 hour incorporation to 282 dpm/μg DNA for 29 hour incorporation, decreased slightly to a minimum of 216 dpm/μg DNA for 51 hour incorporation, and returned to 270 dpm/μg DNA for 87 hour incorporation. Cells incubated at 37°C for 5 days resulted in the amount of "unscheduled DNA synthesis" increasing from 64 dpm/μg DNA for 2 hour incorporation to 285 dpm/μg DNA for 41 hour incorporation. This value decreased to approximately zero at 51 hour incorporation. After 63 hours, more ^{3}H-Thymidine was incorporated into the DNA of non-irradiated cells than in cells that were exposed to ultraviolet irradiation.

The above experiment was repeated except that ^{3}H-Thymidine was incorporated at 37°C for 2, 5, 16, 30, 41, 54, 66, 78, 93 and 116 hours after ultraviolet irradiation. Hydroxyurea was added at 21, 45, 68, and 96 hours after ultraviolet irradiation, as well as 1 hour before irradiation, in increasing increments of 5 m$\underline{M}$ each. The results are shown in Figures 15 and 16.

Although not shown, the amount of DNA isolated averaged 64 μg for plates allowed to incorporate ^{3}H-Thymidine for 2 hours, ranging from 44 to 88 μg per plate. DNA isolated averaged 48 μg for plates

with 41 hour incorporation, ranging from 38 to 60 μg per plate. Finally, DNA isolated averaged 21 μg for plates with 93 hour incorporation, ranging from 8 to 30 μg per plate. Again there was no correlation between the amount of DNA isolated and prior temperature, or whether or not the cells were irradiated.

Figure 15 shows the amount of ^{3}H-Thymidine incorporation per unit weight of DNA as a function of the length of time the ^{3}H-Thymidine was allowed to incorporate. For cells incubated at 33°C for 5 days before ultraviolet irradiation, the mean specific activity was 162 dpm/μg DNA for 2 hour incorporation and increased to 2242 dpm/μg DNA for 41 hour incorporation. This increased slightly to 2303 dpm/μg DNA for 93 hour incorporation. For non-irradiated cells incubated at 33°C for 5 days, the mean specific activity was 65 dpm/μg DNA for 2 hour incorporation and increased to 1340 dpm/μg for 41 hour incorporation. This decreased slightly to 1282 dpm/μg DNA for 93 hour incorporation.

For cells incubated at 37°C for 5 days before ultraviolet irradiation, the mean specific activity was 194 dpm/μg DNA for 2 hour incorporation and increased to 1714 dpm/μg DNA for 41 hour incorporation. The mean specific activity remained fairly constant and then decreased to 922 dpm/μg DNA for 93 hour incorporation. The mean specific activity for non-irradiated cells, incubated at 37°C for 5 days before ^{3}H-Thymidine incorporation was 54 dpm/μg DNA for 2 hour incorporation and increased to 916 dpm/μg DNA for 41 hour incorporation. This decreased to 712 dpm/μg DNA for 93 hour incorporation.

Figure 16 shows the amount of "unscheduled DNA synthesis" attributed only to ultraviolet induced excision repair. The amount of "unscheduled DNA synthesis" in cells incubated for 5 days at 33°C

Figure 15. The amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells at 37°C as a function of the duration of ^{3}H-Thymidine incorporation, following a 120 hour incubation period at either 37°C (circles) or 33°C (squares) and 254 nm ultraviolet irradiation at 15 J/m^{2} (hollow symbols) or non-irradiation (solid symbols). Hydroxyurea ("HU") was added at 21, 45, 68, and 96 hours after ultraviolet irradiation, as well as 1 hour before irradiation, in increasing increments of 5 mM each

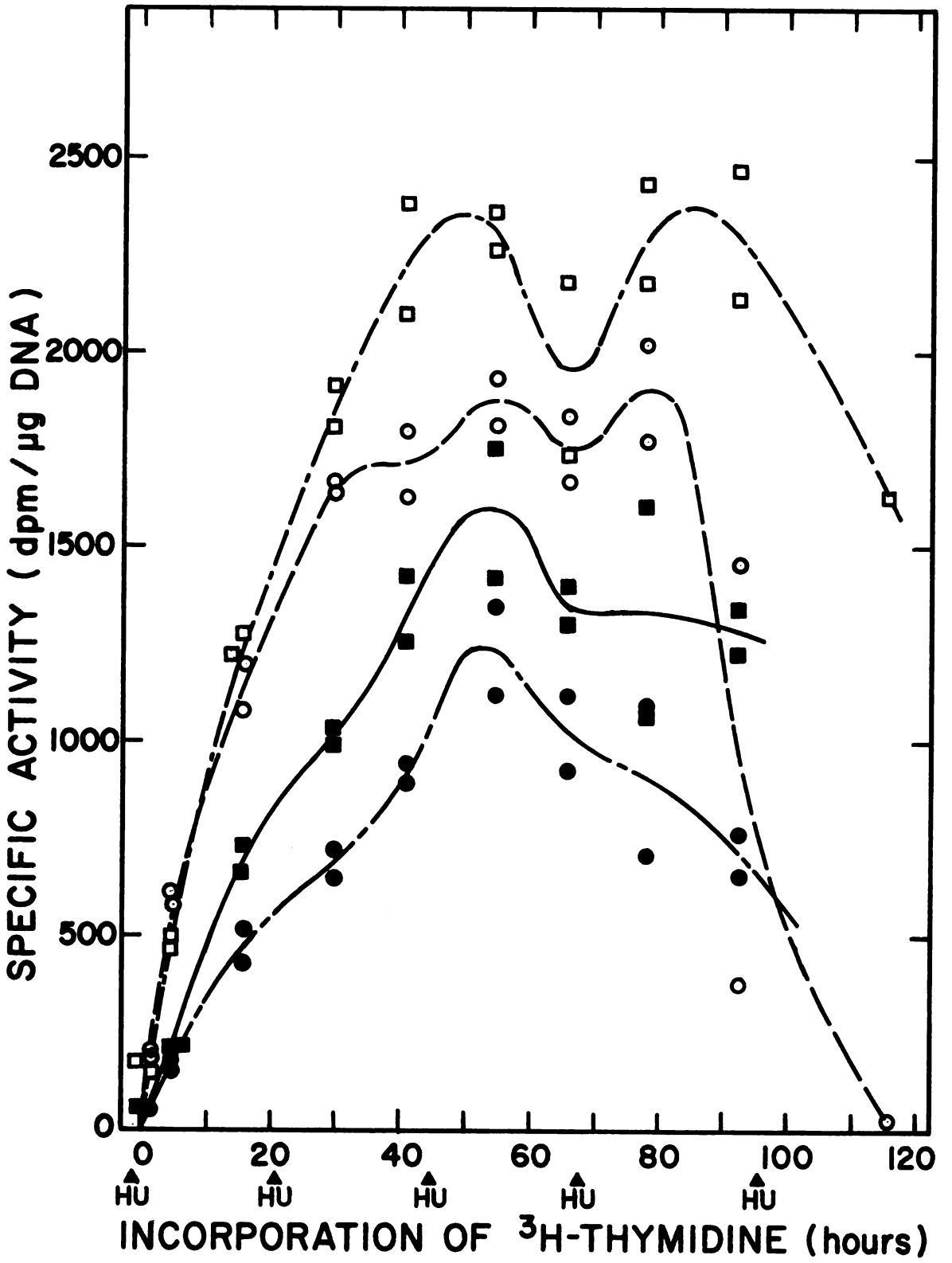

SPECIFIC ACTIVITY (dpm/µg DNA)
2500
2000
1500
1000
500
0
0
20
40
60
80
100
120
HU
HU
HU
HU
HU
INCORPORATION OF ^{3}H-THYMIDINE (hours)

Figure 15.

Figure 16. The net amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of V-79 cells at 37°C, which was used for the excision repair of ultraviolet radiation-induced dimers, as a function of the duration of ^{3}H-Thymidine incorporation. Each point is derived from the difference of the averaged values of points in Figure 15 and Figure 17 between those representing ultraviolet irradiated cells and non-irradiated cells, which were previously incubated for 120 hours (Figure 15) at either 37°C (solid circles) or 33°C (squares) or were previously incubated for 72 hours (Figure 17) at either 37°C (hollow circles) or at 40°C (triangles)

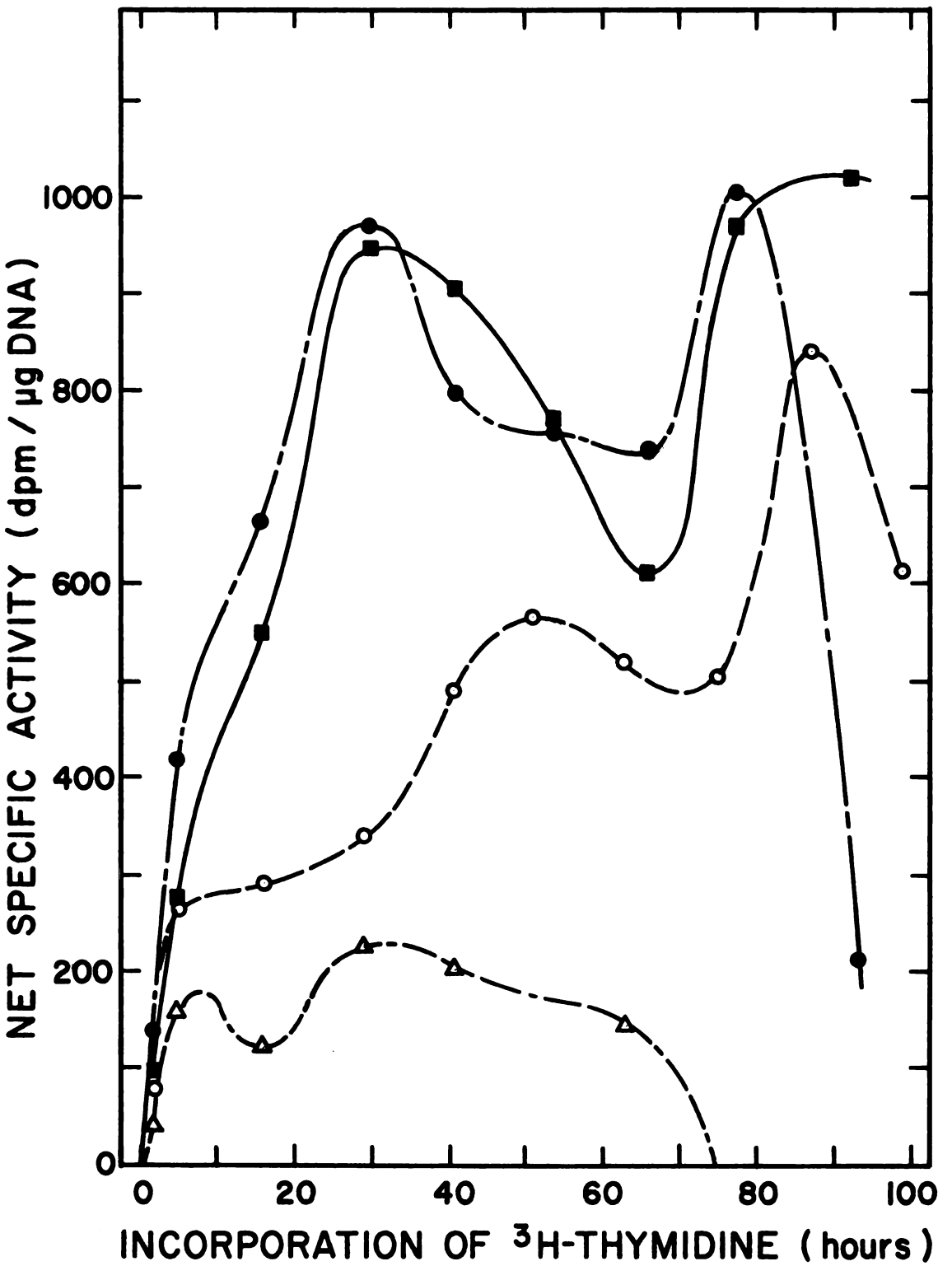

NET SPECIFIC ACTIVITY (dpm / µg DNA)
1000
800
600
400
200
0
0
20
40
60
80
100
INCORPORATION OF ³H-THYMIDINE (hours)

Figure 16.

was 97 dpm/μg DNA for 2 hours, 902 dpm/μg DNA for 41 hours, and 1021 dpm/μg DNA for 93 hours. In cells always incubated at 37°C, "unscheduled DNA synthesis" was 140 dpm/μg DNA for 2 hour incorporation, 798 dpm/μg DNA for 41 hours, and 210 dpm/μg DNA for 93 hours.

The results from both experiments show that pre-UV hypothermia does not appear to affect the amount of "unscheduled DNA synthesis" after UV irradiation when ^{3}H-Thymidine was allowed to incorporate into the DNA for periods up to 41 hours. However, hypothermia for 5 days before ^{3}H-Thymidine incorporation of non-irradiated cells either resulted in an increase, or no change, of incorporation of the radioactive label. This suggests that 5 day hypothermia sometimes, but not always, results in an increase in subsequent residual "semi-conservative DNA synthesis". After 41 hours, ^{3}H-Thymidine uptake into DNA varied with different treatments, but the results are difficult to interpret.

10. <u>The effect of incubation at 40°C for 3 days before ultraviolet irradiation on various periods of "unscheduled DNA synthesis" in V-79 cells</u>.

An experiment was conducted to determine the effect of pre-UV hyperthermia on the amount of "unscheduled DNA synthesis" for various durations after ultraviolet irradiation.

Chinese hamster lung fibroblasts were inoculated into 60 mm plastic Petri dishes and were allowed to grow to confluence at 37°C. At confluence, the medium was changed to one deficient in arginine and isoleucine. At this point, the cells were incubated for 72 hours at either 37°C or 40°C. Hydroxyurea was added after this period at a concentration of 5 m<u>M</u> and the cells were incubated at 37°C for 1

hour. At this point, at least two plates in each temperature group were exposed to ultraviolet radiation and the DNA in all of the plates was allowed to uptake ^{3}H-Thymidine at 37°C for either 2, 5, 16, 29, 41, 51, 63, 75, 87, or 99 hours. Hydroxyurea was added after 18, 42, and 66 hours of ^{3}H-Thymidine uptake, in additions of 5 m$\underline{M}$ each. The results are shown in Figures 16 and 17.

The amount of DNA isolated per plate is illustrated on the top of Figure 17. In cells that were always incubated at 37°C, the average amount of DNA isolated per plate was 108 µg after 2 hours of incorporation of ^{3}H-Thymidine, 74 µg after 41 hours, and 11 µg after 99 hours. For cells incubated at 40°C for 72 hours, the average amount of DNA isolated per plate was 93 µg after 2 hours and 30 µg after 41 hours of ^{3}H-Thymidine incorporation. Cells that were incubated at 40°C for 3 days and were allowed to incorporate ^{3}H-Thymidine at 37°C for 51 hours or greater were almost completely detached from the plates.

The bottom of Figure 17 shows the amount of ^{3}H-Thymidine incorporation per weight of DNA as a function of the length of the incorporation period. For irradiated cells, always incubated at 37°C, the mean amount of ^{3}H-Thymidine incorporated per µg DNA was 132 dpm after 2 hours incorporation, 856 dpm after 41 hours, and 1552 dpm after 87 hours. For non-irradiated cells, always incubated at 37°C, mean ^{3}H-Thymidine incorporation per µg DNA was 48 dpm after 2 hours incorporation, 364 dpm after 41 hours, and 930 dpm after 87 hours. For cells incubated at 40°C for 3 days before ultraviolet irradiation, the mean amount of ^{3}H-Thymidine incorporation per µg DNA was 52 dpm after 2 hours incorporation, 372 dpm after 41 hours, and 45 dpm after 75 hours. For non-irradiated cells incubated at 40°C for 3 days, the

Figure 17. The amount of DNA isolated (top) and ^{3}H-Thymidine (5 µCi/ml medium) incorporated into the DNA (bottom) of V-79 cells at 37°C as a function of the duration of ^{3}H-Thymidine incorporation, following a 72 hour incubation period at either 37°C (circles) or 40°C (triangles) and 254 nm ultraviolet irradiation at 15 J/m^{2} (hollow symbols) or non-irradiation (solid symbols). Hydroxyurea was added at 18, 42, and 66 hours after ultraviolet irradiation, as well as 1 hour before irradiation, in increasing increments of 5 m<u>M</u> each

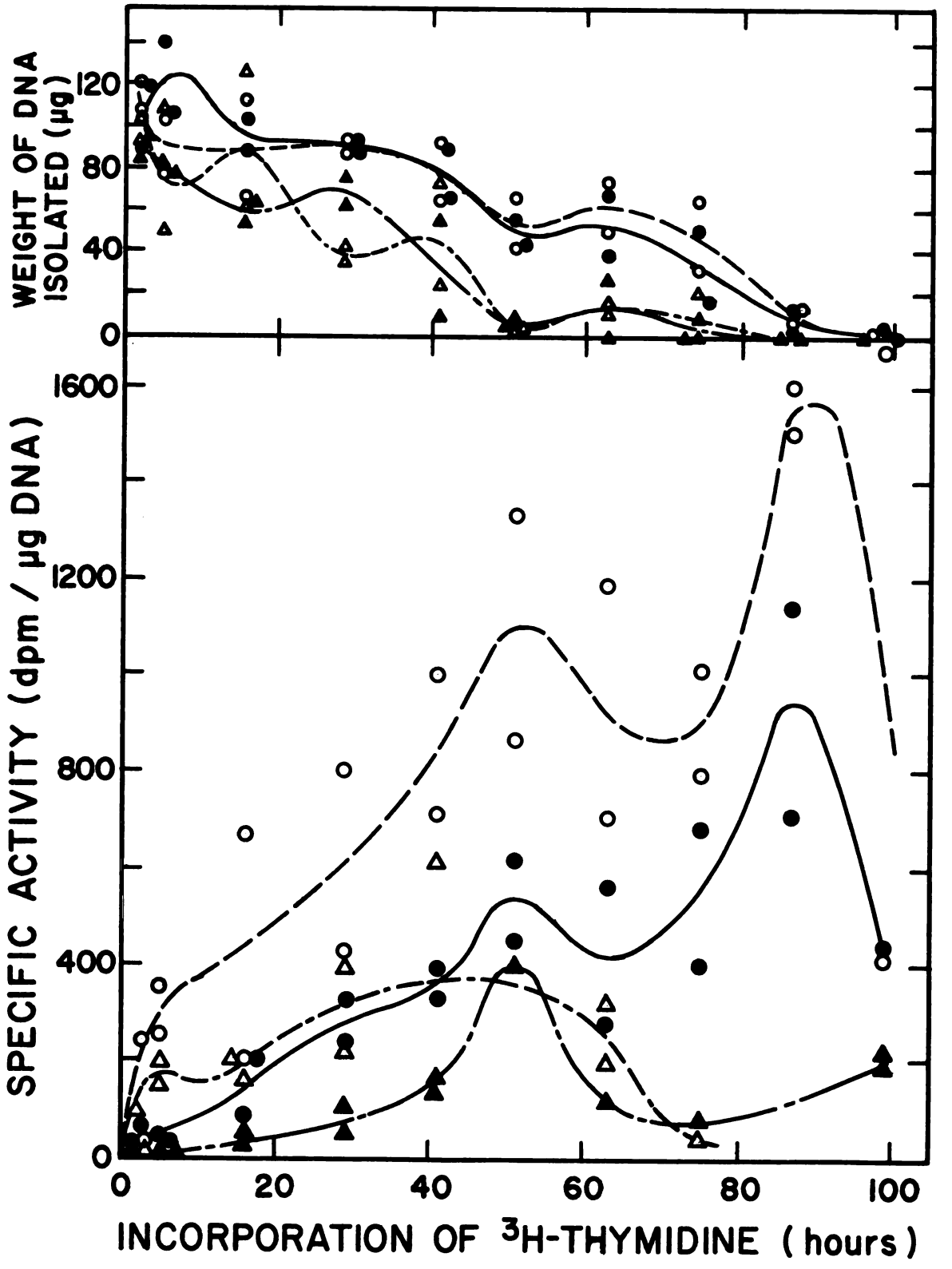

WEIGHT OF DNA ISOLATED (µg)
120
80
40
0
SPECIFIC ACTIVITY (dpm / µg DNA)
1600
1200
800
400
0
0
20
40
60
80
100
INCORPORATION OF ^{3}H-THYMIDINE (hours)

Figure 17.

mean ^{3}H-Thymidine incorporation per µg DNA was 11 dpm after 2 hours incorporation, 171 dpm after 41 hours, and 84 dpm after 75 hours.

Figure 16 shows the amount of "unscheduled DNA synthesis" attributed only to the excision repair of ultraviolet induced dimers. This is the difference of the mean specific activities of ^{3}H-Thymidine incorporation into DNA in ultraviolet irradiated cells and non-irradiated cells. The amount of "unscheduled DNA synthesis" in cells always incubated at 37°C was 85 dpm/µg DNA after 2 hours of ^{3}H-Thymidine incorporation and 492 dpm/µg DNA after 41 hours. The amount of "unscheduled DNA synthesis" was saturated after this point with a specific activity of approximately 500 dpm/µg DNA. The amount of "unscheduled DNA synthesis" in cells incubated at 40°C for 3 days was 41 dpm/µg DNA after 2 hours and saturated at 225 dpm/µg DNA at 29 hours of incorporation. After 41 hours this net specific activity decreased, and reached zero at 75 hours of incorporation.

The results indicate that pre-UV hyperthermia produced a decrease in both the initial rate and maximum extent of "unscheduled DNA synthesis" after UV irradiation. This hyperthermia also resulted in a faster rate of cell detachment from the plates during the period of ^{3}H-Thymidine incorporation.

11. <u>The effect of incubation at 41°C for 3 days before ultraviolet irradiation on 2 and 24 hours of ^{3}H-Thymidine incorporation in human skin fibroblasts</u>.

Human cells were utilized in an experiment to determine the effect of pre-UV hyperthermia on the amount of subsequent "unscheduled DNA synthesis". The different cell type was utilized to show that a reduction of "unscheduled DNA synthesis" with pre-UV hyperthermia is not necessarily restricted to V-79 cells.

Human skin fibroblasts, at passage 20, were inoculated into 60 mm plastic Petri dishes, at a concentration of about 10^6 cells per dish, and were allowed to grow at 37°C for 36 hours to confluence. The confluent cells were incubated in a medium deficient in arginine and isoleucine, for 72 hours, at either 37°C or 41°C. After this period, hydroxyurea was added at a concentration of 5 mM and all the plates were incubated at 37°C for 1 hour. At this point, most of the plates were exposed to ultraviolet irradiation, and all of the cells were then allowed to incorporate ^{3}H-Thymidine at 37°C for either 2 or 24 hours.

The amount of DNA isolated per plate averaged 19.5 µg, ranging from 14.2 to 26.9 µg. The amount of DNA isolated per plate did not appear to be affected by temperature, ultraviolet irradiation, or the period of ^{3}H-Thymidine incorporation. Figure 18 shows the amount of ^{3}H-Thymidine incorporation as a function of the length of time of incorporation. For cells incubated at 37°C before ultraviolet irradiation, the specific activity increased from an average value of 1530 dpm/µg DNA for 2 hour incorporation to 4430 dpm/µg DNA for 24 hour incorporation. For cells incubated at 41°C for 3 days before ultraviolet irradiation, the specific activity increased from average of 840 dpm/µg DNA for 2 hour incorporation to 4210 dpm/µg DNA for 24 hour incorporation. Non-irradiated cells incorporating ^{3}H-Thymidine for 2 hours showed a specific activity of approximately 100 dpm/µg DNA.

Like the experiments utilizing V-79 cells, the above experiment demonstrates that pre-UV hyperthermia results in a significant decrease in subsequent "unscheduled DNA synthesis" in human skin fibroblasts.

Figure 18. The amount of ^{3}H-Thymidine (5 µCi/ml medium) incorporated into the DNA of human skin (736-NF) fibroblasts at 37°C as a function of the duration of ^{3}H-Thymidine incorporation, following a 72 hour incubation period at either 37°C (circles) or 41°C (triangles), hydroxyurea (5 mM) treatment for 1 hour, and 254 nm ultraviolet irradiation at 15 J/m^{2} (hollow symbols) or non-irradiation (solid symbols)

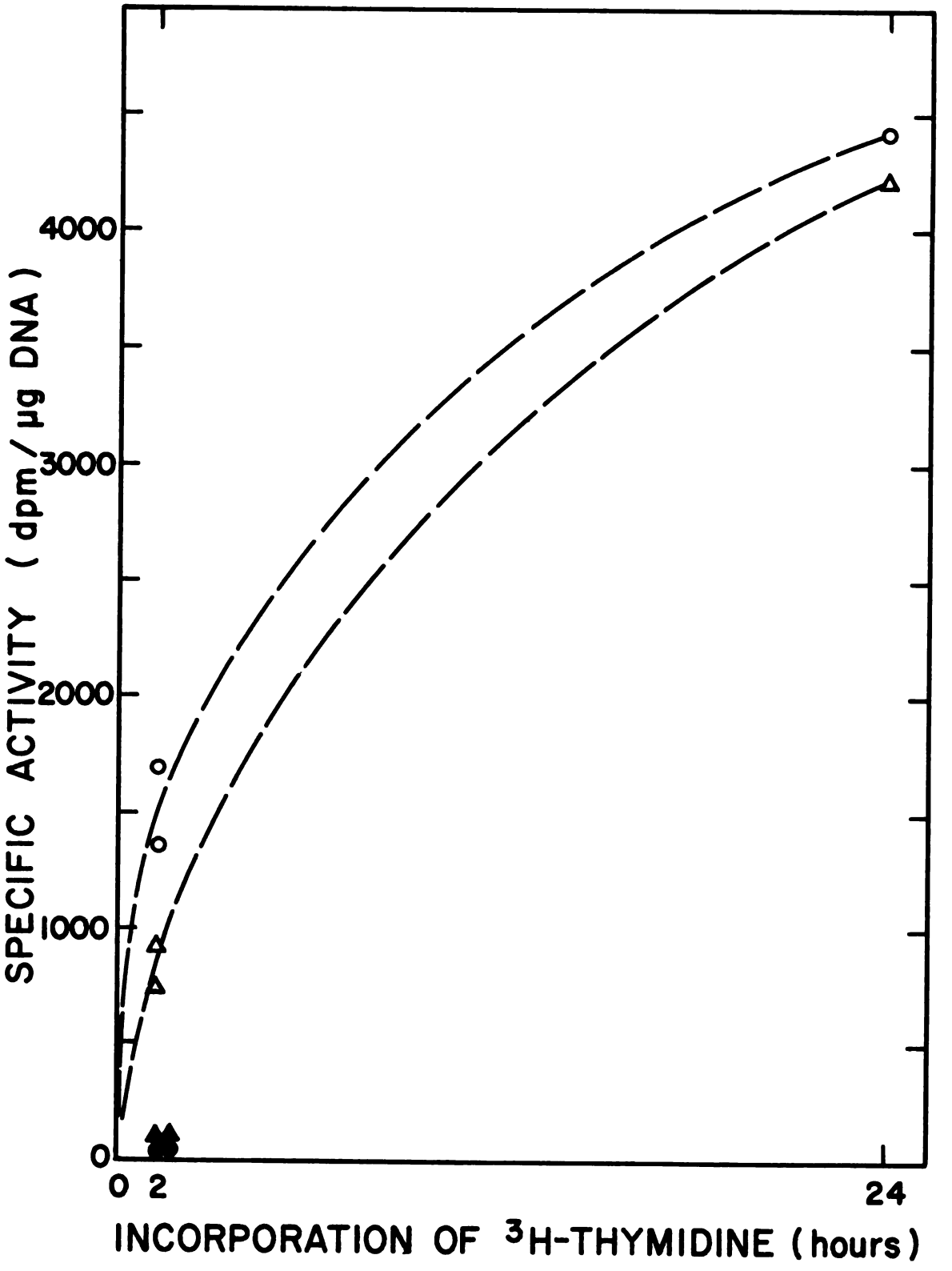

SPECIFIC ACTIVITY (dpm/ µg DNA)
4000
3000
2000
1000
0
0 2
24
INCORPORATION OF 3H-THYMIDINE (hours)

Figure 18.

12. The effect of incubation at 37°C and 41°C for 1, 8, and 24 hours in deficient medium on subsequent "unscheduled DNA synthesis" in human fibroblasts.

In all of the preceding experiments involving the effect of hyperthermia on subsequent "unscheduled DNA synthesis" (Sections 1 - 5, 10, and 11), cells were incubated at the higher temperature for various durations within the 3 day period while being maintained at confluence in deficient medium. During this 3 day period in deficient medium, the cells were proceeding from a state of normal "semi-conservative DNA synthesis" to a state of very limited "semi-conservative DNA synthesis". Many different effects of temperature can take place during this period, which may influence the amount of subsequent "unscheduled DNA synthesis". Therefore an experiment was conducted in which the cells were subjected to a period of hyperthermia following the 3 day period in deficient medium.

Human skin fibroblasts, at passage 22, were inoculated into 60 mm plastic Petric dishes at a concentration of approximately 10^6 cells per dish, and were allowed to grow at 37°C for 41 hours to confluence. At confluence, the cells were maintained at 37°C for 72 hours in a medium deficient in arginine and isoleucine. After this period, the cells were incubated for an additional period of 1, 8, or 24 hours at either 37°C or 41°C. Hydroxyurea was added at a concentration of 5 mM at the beginning of the final 1 hour of this additional period. At this point half the plates were exposed to ultraviolet radiation, and all the cells were allowed to incorporate ^{3}H-Thymidine at 37°C for 2 hours. The amount of DNA isolated per plate averaged 16.7 µg, ranging from 11.8 to 23.6 µg. The amount of DNA isolated did not depend on incubation temperature or exposure

to ultraviolet radiation, but it did decrease slightly with increasing duration of incubation before ^{3}H-Thymidine incorporation. Figure 19 shows the amount of ^{3}H-Thymidine incorporation as a function of the length of the additional period the cells were maintained at 37°C or 41°C in the deficient medium after the initial 72 hour period at 37°C. Cells incubated at 37°C before irradiation had mean specific activities of 2800, 2850, and 1900 dpm/μg DNA, while cells incubated at 41°C before irradiation had mean specific activities of 2800, 2500, and 1870 dpm/μg DNA, for respective additional incubation periods of 1, 8, and 24 hours after the initial 72 hour period at 37°C. Non-irradiated cells always had specific activities of nearly 100 dpm/μg DNA.

The results of this experiment show no significant effect of pre-UV hyperthermia, for periods up to 24 hours, on the amount of subsequent "unscheduled DNA synthesis". However there appeared to be a decrease in "unscheduled DNA synthesis" with increasing duration of pre-UV incubation in deficient medium regardless of the temperature.

DISCUSSION

Ultraviolet irradiated cells always had more ^{3}H-Thymidine incorporation than non-irradiated cells when they were allowed to incorporate for 41 hours or less. When Chinese hamster lung fibroblasts, always incubated at 37°C, were allowed to incorporate ^{3}H-Thymidine for 2 hours, irradiated cells showed from 2.1 (Figure 2) to 4.9 (Figure 4) times more incorporation than non-irradiated ones. Likewise, when human skin fibroblasts, always incubated at 37°C, were allowed to incorporate ^{3}H-Thymidine for 2 hours, irradiated cells showed 15 (Figure 18) to 35 (Figure 19) times more incorporation than non-irradiated cells. This is consistent with previous experiments of J. E. Trosko (personal

Figure 19. The amount of ^{3}H-Thymidine (5 μCi/ml medium) incorporated into the DNA of human skin (736-NF) fibroblasts at 37°C as a function of previous durations of incubation at either 37°C (circles) or 41°C (triangles), after an initial 72 hour incubation period at 37°C. Just prior to ^{3}H-Thymidine incorporation, the cells were treated with hydroxyurea (5 mM) for 1 hour and either exposed (hollow symbols) or not exposed (solid symbols) to 254 nm ultraviolet irradiation at 15 J/m^{2}

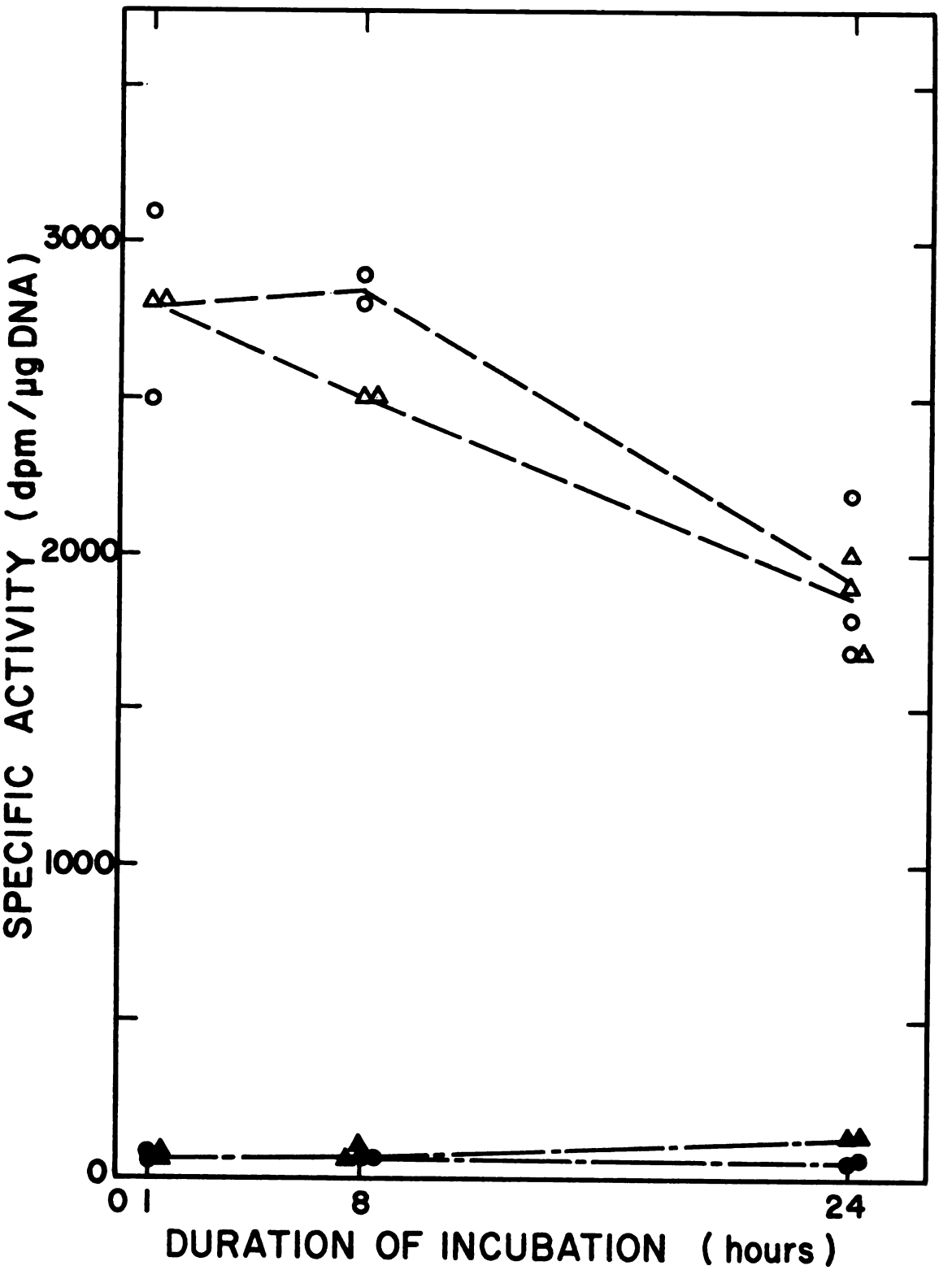

SPECIFIC ACTIVITY (dpm/µg DNA)
3000
2000
1000
0
0 1
8
24
DURATION OF INCUBATION (hours)

Figure 19.

communication) and published results[92]. This indicates that the technique used is sensitive enough to detect "unscheduled DNA synthesis" involved in the excision repair of ultraviolet induced damage. These results also indicate that the amount of "unscheduled DNA synthesis" is much greater in human skin fibroblasts than in Chinese hamster lung fibroblasts for the given ultraviolet radiation dose of 15 J/m^2.

Although Chinese hamster cells that were incubated at 43°C for 2 hours were almost completely detached, cells incubated at 40°C for 2 hours incorporated only slightly less ^{3}H-Thymidine than cells that were always incubated at 37°C (Table 1). This decrease did not appear to be significant. Also, V-79 cells incubated at 41°C for 8 hours showed an insignificant increase (Figure 2) or decrease (Figure 3) of ^{3}H-Thymidine incorporation with respect to cells always incubated at 37°C. Although this deviation appears insignificant, it might depend on whether the growth time for the cells to reach confluence was 3 days (Figure 2) or 4 days (Figure 3). The same cells incubated at 41°C for 21 hours always had less ^{3}H-Thymidine incorporation for 2 hours than cells constantly maintained at 37°C. Although this decrease was insignificant for the experiment displayed in Figure 2, cells incubated at 41°C for 21 hours in the experiment shown in Figure 3 resulted in a net specific activity of 2 hour incorporation due to UV irradiation alone of 50 dpm/μg DNA, which is 67% of the net specific activity of 75 dpm/μg DNA observed for cells that were only incubated at 37°C.

Hyperthermia for 3 days prior to UV irradiation always resulted in significantly less ^{3}H-Thymidine incorporation. This was demonstrated in 6 experiments illustrated in Figures 4 - 8 and 16 - 18. Chinese

hamster cells incubated at 41°C for 3 days resulted in the net specific activity being between 23% (Figure 8) and 60% (Figure 5) of the value derived from cells only incubated at 37°C with 2 hour incorporation. The same cells incubated at 40°C for 3 days had 49% of ^{3}H-Thymidine incorporated for 2 hours as in cells only incubated at 37°C (Figure 16). Human skin fibroblasts incubated at 41°C for 3 days had a net specific activity which was 50% of that from cells only incubated at 37°C (Figure 18).

Chinese hamster cells, incubated at 41°C for 3 days before hydroxyurea treatment and allowed to incorporate ^{3}H-Thymidine at 37°C for 2 hours, gave a net specific activity of 50 dpm/μg DNA when incubated with hydroxyurea at 37°C for 1 hour, and 32 dpm/μg DNA at 41°C. This difference does not appear to be significant. At least, incubation at 41°C for 1 hour does not appear to break down the hydroxyurea, resulting in more ^{3}H-Thymidine incorporation from "semi-conservative DNA synthesis".

The same cells, incubated at 41°C for 3 days and at 41°C for 1 hour with hydroxyurea, gave a net specific activity of 32 dpm/μg DNA when ^{3}H-Thymidine was incorporated for 2 hours at 37°C after ultraviolet irradiation, and 70 dpm/μg DNA when incorporation was done for 2 hours at 41°C after irradiation. It is well known that the rates of many enzymatic reactions increase with higher temperature. The observed increase in specific activity, when ^{3}H-Thymidine was incorporated at the higher temperature, might be due to an increased rate of enzymatic processes involved in the excision of ultraviolet induced dimers. As was previously discussed, a significant reduction in ^{3}H-Thymidine incorporation was observed only when

cells were pre-UV incubated at 41°C for periods longer than 21 hours. Certainly any thermal impairment of the excision process, such as denaturation of the excision repair enzymes, would be negligible during the 2 hour incorporation period at 41°C.

As shown in Figure 6, cycloheximide does not appear to affect the amount of ^{3}H-Thymidine incorporated into the DNA, for irradiated or non-irradiated cells, incubated at either 37°C or 41°C for 3 days. Apparently maintenance of V-79 cells in a medium deficient in arginine and isoleucine is enough to halt protein synthesis. Cycloheximide is not needed to prevent repair proteins from being resynthesized and was not used in subsequent experiments.

No definite conclusions could be reached from experiments involving incubation of V-79 cells at 33°C for periods up to 5 days before ^{3}H-Thymidine incorporation (Figures 9 - 16), except that this period of pre-UV hypothermia does not appear to affect the net amount of "unscheduled DNA synthesis" attributed only to the excision repair of ultraviolet induced damage, when incorporation was conducted for 41 hours or less. This hypothermia sometimes increases (Figures 12 - 15), decreases (Figure 10), or apparently has no definite effect (Figures 9, 11, and 16) on total incorporation of ^{3}H-Thymidine at 37°C for 2 hours. Since this data is inconsistent, it could only be concluded that hypothermia before ultraviolet irradiation had no significant effect or that the effects are too complicated to be interpreted.

According to Figures 14 and 16, net ^{3}H-Thymidine incorporation for excision repair is saturated at approximately 27 hours. Saturation was observed with other cell types by other investigators[2]. However, as shown in Figure 16, cells incubated at 40°C for 3 days were saturated

with ^{3}H-Thymidine at 16 hours, while the cells that were used in the same experiment, but were only incubated at 37°C, saturated at 41 hours. In this experiment, there was less DNA isolated at saturation, than at times before saturation, for cells incubated at both temperatures (Figure 17). This suggests that cells were detaching, and perhaps dying, before ^{3}H-Thymidine saturation.

From the top of Figure 17, it appears that cells incubated at 40°C for 3 days were detaching (or dying) at a much faster rate than cells only incubated at 37°C. This observation supports the hypothesis that thermal denaturation of the repair processes is the rate limiting step in the death of single cells. The argument goes as follows. Incubation at 40°C for 3 days denatures the repair processes so that the cells could perhaps repair only half as well as cells that were always incubated at 37°C. The denaturation of a repair process would be a "rate limiting step", since it takes 3 days, a relatively long time, to accomplish. Next, environmental insults, such as ultraviolet irradiation, damage the DNA. These are obviously not "rate limiting steps", since they occur within a fraction of a second. After 2 hours of incorporation of ^{3}H-Thymidine at 37°C, almost the same amount of DNA was isolated for cells incubated for 3 days at either 37°C or 40°C, but cells incubated at 40°C incorporated only 49% of ^{3}H-Thymidine as cells only incubated at 37°C. After 2 hours, the cells did not accumulate enough unrepaired damage, or the damage did not have a sufficient period of time to cause cell mortality, yet the excision repair of UV induced dimers in cells incubated at 40°C was only half as efficient as repair in cells incubated at 37°C.

With further incubation at 37°C with ^{3}H-Thymidine, enough unrepaired damage (not necessarily UV induced) would accumulate, within a sufficient period of time, to express its lethality. Cells that were incubated at 40°C would incorporate less ^{3}H-Thymidine, and accumulate unrepaired damage at a faster rate, causing an increased death rate, during the incorporation period, over cells that were only incubated at 37°C. This is observed in the top of Figure 17, if the amount of DNA isolated per plate can be interpreted as a measurement of cell survivorship. The figure also indicates that the cell death rate is not significantly enhanced by prior ultraviolet irradiation, since irradiated cells and non-irradiated cells, kept under otherwise identical conditions, contain almost equal amounts of DNA. This suggests that ultraviolet irradiation is not a major cause of the observed loss of cells. Other damaging causes might be the exposure to 4°C (on ice) before ^{3}H-Thymidine incorporation, continued exposure to radioactive thymidine, or continued maintenance in a spent arginine and isoleucine deficient medium supplemented with dialysed fetal calf serum. These damages are probably repaired by mechanisms other than the pyrimidine dimer excision repair system. Presumably these other mechanisms can be thermally impaired by 72 hour incubation at 40°C. The hypothesis that the denaturation of repair proteins is the rate limiting step in the thermal death of single cells is still consistent with the above argument and observations in Figures 16 and 17. Even though the major cause of cell death is probably not the denaturation of the repair enzymes that excise ultraviolet induced pyrimidine dimers, measuring the repair of these enzymes can be used to monitor the repair by hypothesized repair enzymes that cause cell death if thermally denatured.

Probably exposure to 40°C or greater, over a 3 day period, causes thermal damage to many systems within the cell. These may include repair systems so essential to the cell that the denaturation of repair proteins, contained within these systems, may cause cell death.

However, if thermal cell death caused decreased repair, which is the converse of the above hypothesis, entirely different results would have been observed. First of all a significant amount of cells would have been expected to detach during 3 days of incubation at 40°C as for the cells that were incubated at 43°C for 2 hours (Table 1). This is not observed, since even after 2 hours of ^{3}H-Thymidine incorporation, plates incubated at either 37°C or 40°C contained equal amounts of DNA (Figure 17). However, if in this case "dead" cells did not detach after 3 days at 40°C and if the "dead" cells had not incorporated ^{3}H-Thymidine, while the "live" cells incorporated as much ^{3}H-Thymidine as cells only incubated at 37°C, then the average specific activity for cells incubated at 40°C should be significantly less than for cells only incubated at 37°C. However, the cells incubated at 40°C began to detach after 2 hours of ^{3}H-Thymidine incorporation. The "live" cells have no reason to detach at this point and the "dead" cells, which are not metabolizing, are not expected to maintain their adhesion to the surface. While the "dead" cells detach, the surface of the plate should contain a greater percentage of "live" cells which have as much ^{3}H-Thymidine incorporated into their DNA as cells only incubated at 37°C. With increasing time of incubation with ^{3}H-Thymidine, the DNA isolated should have had greater specific activities, eventually reaching the specific activity of the cells incubated at 37°C,

when the "dead" cells had completely detached. This is clearly not what is observed. Between 16 and 41 hours of ^{3}H-Thymidine incorporation, the period of greatest cell detachment for cells that were incubated at 40°C for 3 days, the specific activity for these cells remained constant, while the specific activity for cells only incubated at 37°C continued to increase. Therefore, the lower specific activity observed in cells incubated at 40°C for 3 days relative to cells always incubated at 37°C appears to be attributable to thermal impairment of the excision repair process rather than to an increasing percentage of dead cells.

As mentioned earlier in the Discussion section, pre-UV hyperthermia for 3 days always significantly reduced the amount of subsequent ^{3}H-Thymidine incorporation into the DNA of both Chinese hamster cells (Figures 4 - 8, 16, and 17) and human skin fibroblasts (Figure 18). Since the period of pre-UV hyperthermia is relatively long, the rate limiting step in the thermal inhibition of ^{3}H-Thymidine incorporation into DNA should be an activated process. The results of the experiment shown in Figure 19 suggest that there may be another time dependent process involved in the inhibition of ^{3}H-Thymidine incorporation. However this process is not an activated process, since an increase in temperature from 37°C to 41°C would not cause any significant further inhibition of ^{3}H-Thymidine incorporation. Following a 3 day period of confluence in deficient medium at 37°C, human skin fibroblasts, taken from the experiments that are illustrated in Figure 19, were further incubated at either 37°C or 41°C for various periods of time prior to ultraviolet irradiation. Although there was no significant effect with temperature on the subsequent incorporation of

^{3}H-Thymidine, a significant reduction in the uptake of this radioactive label was observed with increasing durations of pre-UV incubation, regardless of the temperature. No significant effect with temperature was expected since the period of hyperthermia only extended up to 24 hours. Significant decreases in ^{3}H-Thymidine incorporation were observed only with a 3 day period of pre-UV hyperthermia in previous experiments. This thermally activated process of ^{3}H-Thymidine incorporation may involve the denaturation of a protein, since protein denaturation is an activated process[3]. On the other hand, the time dependent, but temperature independent, inhibition of ^{3}H-Thymidine incorporation in the experiment of Figure 19 is not an activated process, and probably does not involve a protein denaturation. This process of inhibition may involve another process involved in the transport and phosphorylation of exogenous ^{3}H-Thymidine to ^{3}H-dTTP into the DNA of the cell, such as the permeability of the cell membrane to ^{3}H-Thymidine. This might involve a time dependent, but temperature independent, clogging of the membrane with material, resulting in a decrease in permeability of the membrane to ^{3}H-Thymidine.

SUMMARY

Confluent mammalian fibroblasts were incubated for three days at conditions unfavorable for semi-conservative DNA replication. After exposure to 15 J/m^{2} of ultraviolet radiation, the cells were allowed to incorporate ^{3}H-Thymidine. The cells were incubated at different temperatures for various durations immediately before ultraviolet irradiations. Excision repair was quantitated by the amount of ^{3}H-Thymidine incorporated into the DNA after ultraviolet irradiation.

Ultraviolet irradiated cells always had more ^{3}H-Thymidine incorporation than non-irradiated cells. Hyperthermia at 40°C or 41°C, for 3 days before ultraviolet irradiation, always resulted in significantly less ^{3}H-Thymidine incorporation following irradiation. However, after Chinese hamster cells were incubated at 41°C for 3 days and then UV irradiated, more ^{3}H-Thymidine was incorporated into DNA for 2 hours at 41°C than was incorporated for 2 hours at 37°C, indicating that incorporation of the label occurred at a faster rate during hyperthermia. Furthermore, incubation at 41°C for one hour does not appear to break down hydroxyurea. Cycloheximide does not appear to affect the amount of ^{3}H-Thymidine incorporation. However, no definite conclusions could be reached from experiments involving incubation of V-79 cells at 33°C for periods up to 5 days before ^{3}H-Thymidine incorporation.

Net ^{3}H-Thymidine incorporation for excision repair appears to be saturated at approximately 27 hours. Chinese hamster cells that were incubated at 40°C for three days before ^{3}H-Thymidine incorporation detached (died) at a faster rate during the labeling period than cells only incubated at 37°C. Ultraviolet irradiated cells did not appear to detach at a faster rate than non-irradiated cells. These observations are consistent with the hypothesis that thermal denaturation of the repair process is the rate limiting step in the death of single cells. The results expected if the converse of this hypothesis were valid are not observed.

CHAPTER III

THE EFFECT OF TEMPERATURE ON THE REPAIR OF ULTRAVIOLET INDUCED PYRIMIDINE DIMERS IN THE DNA OF MAMMALIAN CELLS AS MEASURED BY AUTORADIOGRAPHY

INTRODUCTION

Autoradiography is a technique used to quantitate the amount of radioactivity present within individual cells or cell organelles. The radioactive cellular material is usually fixed on a microscope slide, exposed with photographic emulsion, developed, and stained. During exposure, nuclear disintegration materials produce "grains" or "tracks" detected in the emulsion. The number of "grains" can be counted in each cell or organelle, which gives the relative amount or radioactivity for each unit.

Autoradiography is usually used to study DNA excision repair in more detail. It is still considered a method of "unscheduled DNA synthesis" because radioactive thymidine is commonly utilized to be incorporated into the DNA when "semi-conservative DNA synthesis" is repressed. Rather than measure the bulk "excision repair" for an entire plate of cells, the autoradiography of "unscheduled DNA synthesis" can be used to measure the relative amounts of "excision repair" within each individual cell or nucleus. This technique was utilized to render more support for the hypothesis that denaturation of the DNA repair proteins is the rate limiting step in the death of single cells.

MATERIALS AND METHODS

Cells

Chinese hamster lung fibroblasts (V-79) were used in the experiment. The cell culture was obtained from J. E. Trosko, Human Development Department, Michigan State University, East Lansing, MI., and was grown under humidified 5% CO_2 in air.

Media

Permanent stock cultures of the Chinese hamster cells utilized a "C-15" medium (see Appendix A) supplemented with 5% fetal calf serum. Growing cell cultures, for the experiment, utilized "D" medium (see Appendix A) supplemented with 5% fetal calf serum. The confluent cells utilized for experiments were maintained in "D" medium without arginine or isoleucine (see Appendix A) and supplemented with 5% dialysed fetal calf serum. All media were supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and mycostatin (100 units/ml).

Experimental Culture Procedure (Figure 1)

Cells were inoculated into 60 mm plastic Petri dishes (Corning Glass Works, Corning, NY) and allowed to grow in "D" medium for 70 hours to heavy confluent densities. Cells were maintained for 72 hours at confluence, at either 37°C or 41°C, in a "D" medium deficient in arginine and isoleucine and supplemented with 5% dialyzed fetal calf serum. This medium was changed to fresh deficient medium after 24 hours of confluence. After the 72 hour period, the cells were incubated at 37°C for 1 hour with 5 mM hydroxyurea.

Ultraviolet Irradiation and Repair

The medium was decanted from the Petri dishes and saved just prior to ultraviolet irradiation. The tops were removed from the dishes and

the cells were exposed to 254 nm ultraviolet radiation, delivered from one germicidal lamp (General Electric, G15 T8) mounted in a sterile transfer hood. The incident dose rate, measured with a short wave Blak-ray meter (Ultraviolet Products, San Gabriel, Calif.) was approximately 1.0 W/m^2. While one of the dishes was being irradiated, the remainder of the dishes were kept on ice (4°C).

[Me-^{3}H]-Thymidine (^{3}H-TdR; New England Nuclear, Boston, Mass.; 40 Ci/mmol) was added to the spent, decanted medium at 5 μCi/ml medium. This radioactive medium was then added to the cells immediately after ultraviolet irradiation. The cells were allowed to incorporate ^{3}H-Thymidine from this medium for 2 hours at 37°C.

After this post-treatment incubation period, the medium was decanted and the cells were washed twice with cold phosphate buffered saline. Their detachment from the dishes was facilitated by a rubber policeman in phosphate buffered saline. The detached cells were collected by centrifugation with the phosphate buffered saline decanted.

Autoradiography

After centrifugation, the pelleted cells were fixed overnight, without disrupting the pellet, in methanol/acetic acid (3:1 v/v). After fixation, the fixative was decanted and the cell pellets were disrupted in methanol/acetic acid (3:1). Next, one or two drops of the cell suspension were added to wet microscope slides covered with distilled water. This allowed the cells to spread in a thin flat film over the slides.

After drying overnight, the cells were dipped in undiluted NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). After an 8 day exposure period, the emulsions were developed for 2 minutes in Kodak

D19 developer, rinsed in double distilled water for 0.5 minute, fixed with Kodak rapid fix for 2 minutes, and rinsed 3 times with double distilled water for 7 - 10 minutes each time.

Staining

The slides were stained with hematoxylin and eosin. After development, the slides were rinsed in distilled water for 2 minutes, stained with hematoxylin for 5 minutes, dipped in distilled water, and dipped in tap water. After rinsing with 35%, 50%, 70%, and 95% ethanol for 2 minutes each, the slides were stained with eosin for 2 minutes. At this point, the slides were dipped in 95%, another 95%, and 100% ethanol, and rinsed in two solutions of xylene for 4 minutes each. Finally cover slipes were mounted on the slides with Permount.

RESULTS

As shown in the autoradiographs in Figure 20 and Figure 21, cells that were only incubated at 37°C (Figure 20) appear to contain many more grains per nucleus than cells that were incubated at 41°C for 3 days (Figure 21). Figure 22 shows the results of counting the number of grains per nucleus for 700 cells in each treatment group. This histogram shows the number of nuclei having the number of grains indicated on the x-axis. This indicates that cells only incubated at 37°C tended to contain significantly more grains per nucleus than cells incubated at 41°C for 3 days.

The total amount of grains counted was 15,548 in the 700 cells that were only incubated at 37°C and 5,321 in the 700 cells that were incubated at 41°C for 3 days. This resulted in an average of 22.2 grains per nucleus for cells only incubated at 37°C and 7.6 grains per nucleus for

Figure 20. Autoradiograph of V-79 nuclei after 72 hour incubation at 37°C, treatment for 1 hour with hydroxyurea (5 m$\underline{M}$), ultraviolet irradiation (254 nm) at 15 J/m^2, and exposure to ^{3}H-Thymidine (5 µCi/ml medium) for 2 hours at 37°C

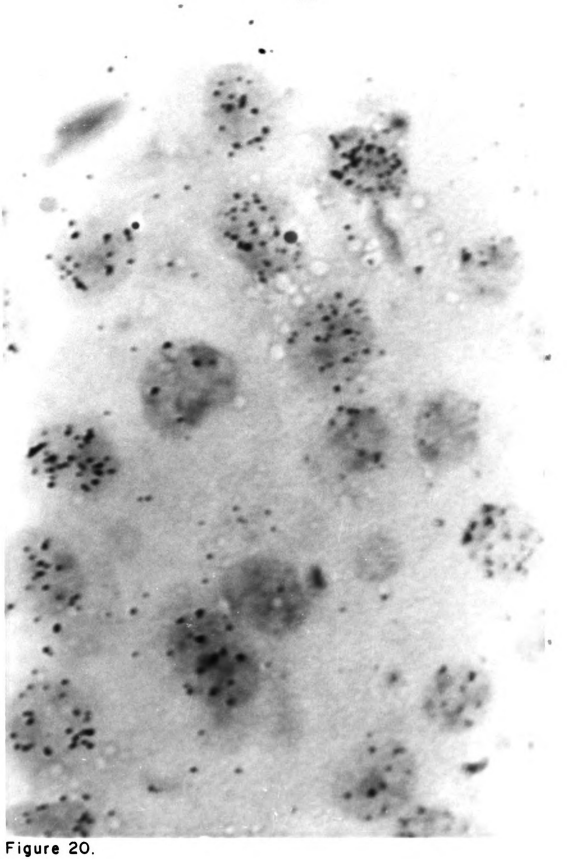


Figure 20.

Figure 21. Autoradiograph of V-79 nuclei after 72 hour incubation at 41°C, treatment for 1 hour with hydroxyurea (5 mM), ultraviolet irradiation (254 nm) at 15 J/m^2, and exposure to ^3H-Thymidine (5 µCi/ml medium) for 2 hours at 37°C

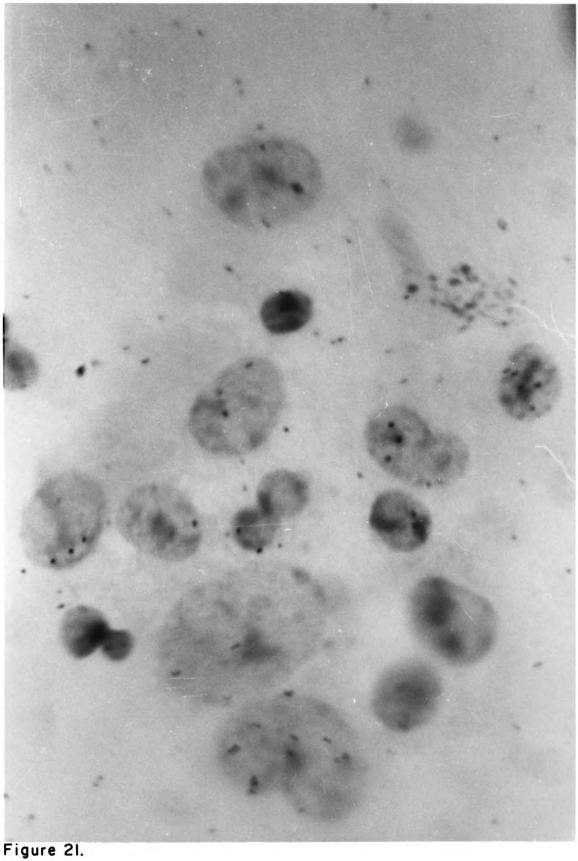


Figure 21.

Figure 22. Distribution curves representing the number of cells with indicated grains per nucleus. V-79 cells were incubated at either 37°C (solid circles) or 41°C (hollow circles) for 72 hours in a medium deficient in arginine and isoleucine, treated for 1 hour with hydroxyurea (5 m$\underline{M}$), ultraviolet irradiated (254 nm) at 15 J/m^2, and exposed to ^{3}H-Thymidine (5 µCi/ml medium) for 2 hours. Seven hundred cells were counted in each treatment group. The absence of a data point means there were no cells with that grain number

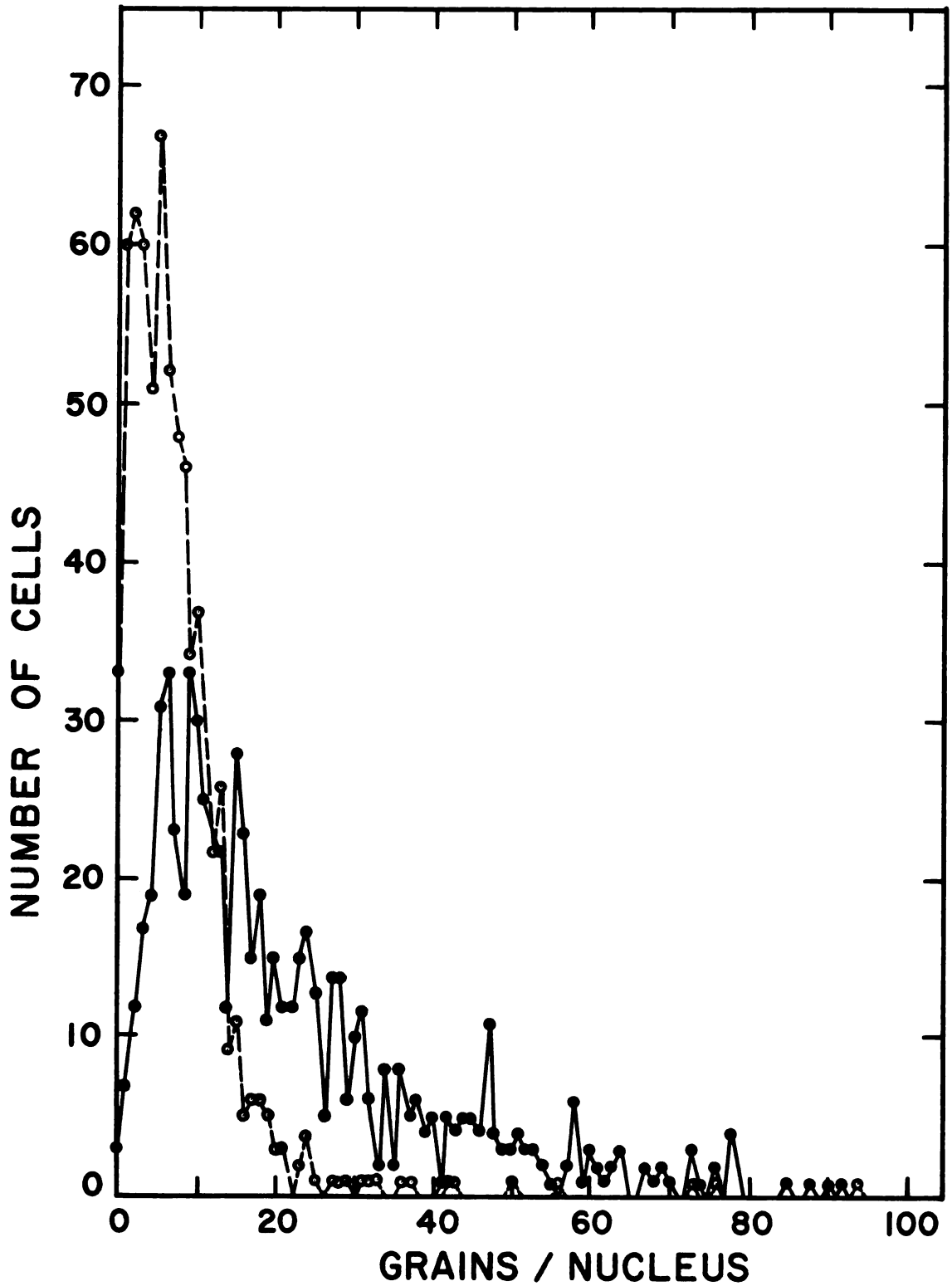

NUMBER OF CELLS
70
60
50
40
30
20
10
0
0
20
40
60
80
100
GRAINS / NUCLEUS

Figure 22.

cells incubated at 41°C for 3 days. If each cell contained an average of 6 pg DNA[97], the specific activities were 321 grains per minute per µg DNA for cells only incubated at 37°C and 110 grains per minute per µg DNA for cells incubated at 41°C for 3 days. Nuclei from cells that were only incubated at 37°C contained, on the average, 2.92 times more grains than nuclei from cells incubated at 41°C for 3 days.

DISCUSSION

Cells only incubated at 37°C incorporated an average of roughly 3 times more ^{3}H-Thymidine in their nuclei than cells incubated at 41°C for 3 days before ultraviolet irradiation. This result can be explained by two hypotheses: Either the 3 days incubation at 41°C causes the cells to incorporate 33% as much ^{3}H-Thymidine as cells only incubated at 37°C or 67% of the cells incubated at 37°C were killed by exposure at 41°C for 3 days before ultraviolet irradiation. Figure 23 shows the experimental data again for cells incubated at 41°C for 3 days before ultraviolet irradiation as well as the expected results from the cells incubated at 37°C if either of the hypotheses were correct.

If the cells that were incubated at 41°C for 3 days incorporated 33% as much ^{3}H-Thymidine as cells only incubated at 37°C, the sum of the total number of cells only incubated at 37°C containing either 3n - 1, 3n, or 3n + 1 grains per nucleus should equal the total number of cells containing n grains per nucleus. These hypothetical results are produced from each group of 3 adjacent points on Figure 22 for cells only incubated at 37°C. As shown in Figure 23, these hypothetical results closely match the experimental data.

If incubation at 41°C for 3 days before ultraviolet irradiation killed 67% of the cells that would have been alive if they were only

Figure 23. Distribution curves representing the numbers of cells with indicated grains per nucleus. The solid circles represent the experimental data from Figure 22 for V-79 cells which were incubated at 41°C for 72 hours before ultraviolet irradiation. The hollow circles represent the expected values if the cells incorporated 33% as much ^{3}H-Thymidine as cells that were incubated at 37°C for 72 hours before ultraviolet irradiation. The triangles represent the expected values if 67% of the cells died and incorporated no ^{3}H-Thymidine, while the remaining 33% of the cells incorporated as much ^{3}H-Thymidine as cells that were incubated at 37°C for 72 hours before ultraviolet irradiation

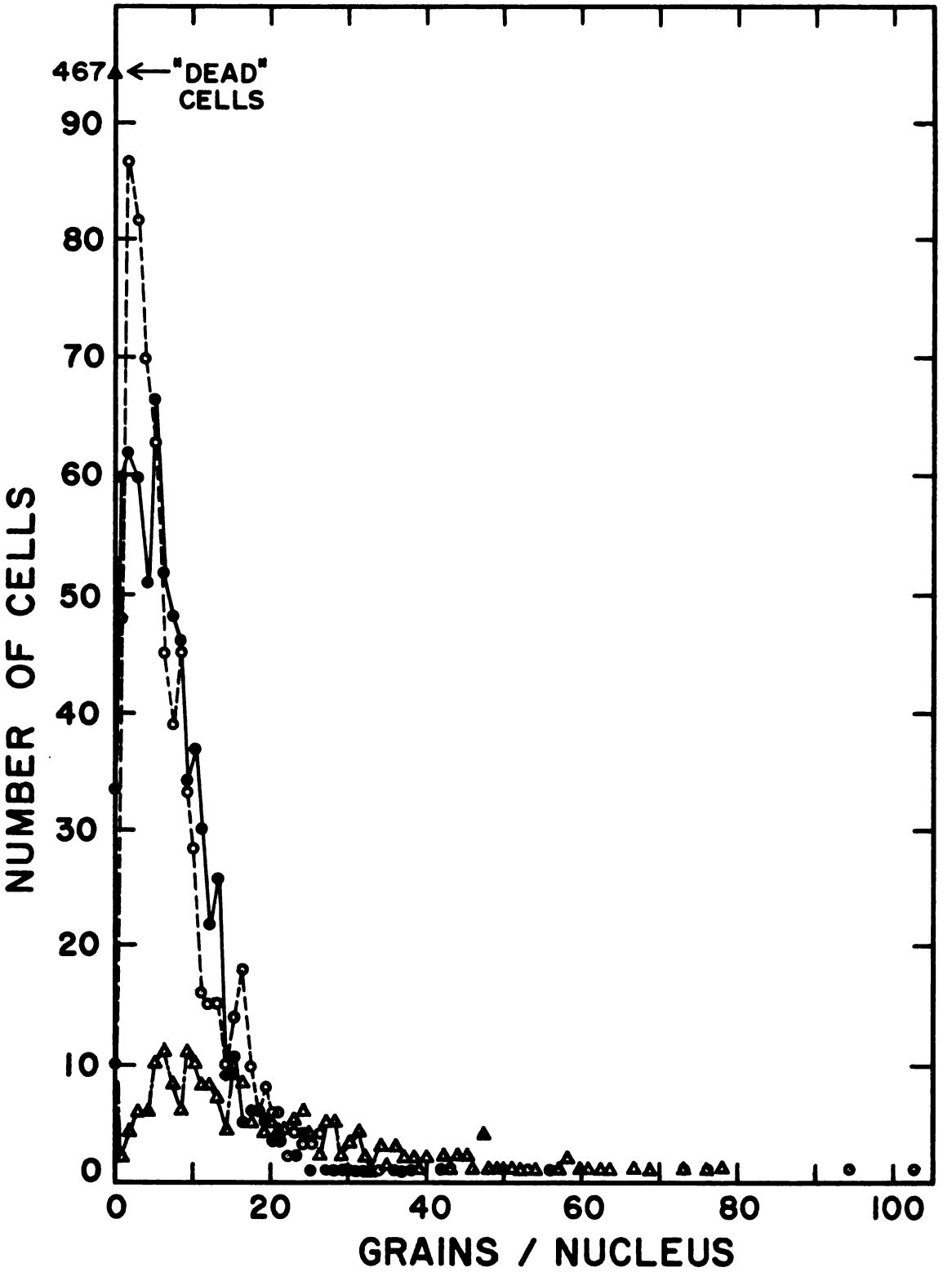
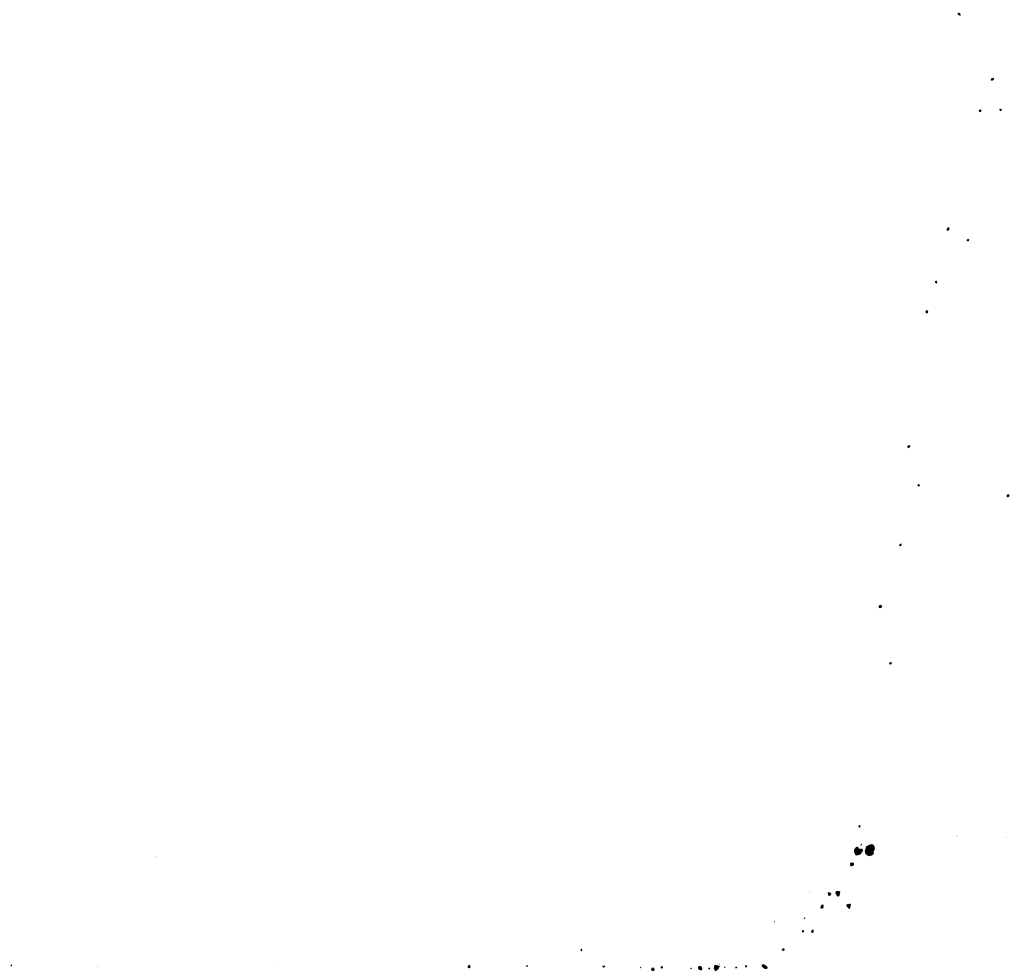

467
"DEAD" CELLS
90
80
70
60
50
40
30
20
10
0
NUMBER OF CELLS
0
20
40
60
80
100
GRAINS / NUCLEUS

Figure 23.



incubated at 37°C, the 467 of the 700 total cells should contain no grains in their nuclei since they are "dead" and therefore should not incorporate ^{3}H-Thymidine. The 233 remaining cells should be distributed like the cells incubated only at 37°C, shown in Figure 22, since these cells should incorporate as much ^{3}H-Thymidine as cells only incubated at 37°C. The number of cells containing a given number of grains per nucleus in the hypothetical results should be 33% of the number of cells containing the same number of grains per nucleus as for cells only incubated at 37°C. As shown in Figure 23, these hypothetical results do not fit the experimental data.

The results of this experiment strongly support the hypothesis that the denaturation of the DNA repair enzymes is a rate limiting step in the death of single cells. Incubation at 41°C would cause a sufficient number of DNA repair proteins to denature so that only 33% of the ^{3}H-Thymidine would be incorporated into the DNA of these cells as compared to cells that were only incubated at 37°C. The actual results obtained would be expected, with the cells dying at a later time.

SUMMARY

Chinese hamster lung fibroblasts were incubated at either 37°C or 41°C for 3 days before ultraviolet irradiation. Incorporation of ^{3}H-Thymidine for 2 hours was measured by autoradiography. The results indicate that nuclei from cells incubated only at 37°C each contain approximately 3 times as much ^{3}H-Thymidine as cells incubated at 41°C for 3 days. This supports the hypothesis that the denaturation of the DNA repair proteins is the rate limiting step in the thermal death of single cells.

CHAPTER IV

THE EFFECT OF TEMPERATURE ON THE REPAIR OF ULTRAVIOLET INDUCED PYRIMIDINE DIMERS IN THE DNA OF MAMMALIAN CELLS AS MEASURED BY ENDONUCLEASE SITE SPECIFICITY

INTRODUCTION

An endonuclease from Micrococcus luteus has been used to monitor the progress of DNA excision repair in ultraviolet irradiated human cells [61]. This endonuclease is utilized in a sensitive enzymatic assay for quantitating the occurrence of pyrimidine dimers in human DNA irradiated in vivo. The endonuclease purified from M. luteus selectively produces single-strand breaks at dimer-containing sites (or nuclease-susceptible sites) in UV-damaged native DNA during the in vitro assay. The amount of endonuclease-induced single-strand breaks is determined by subsequent sedimentation of the endonuclease-treated DNA through alkaline sucrose gradients. The number of UV-induced pyrimidine dimers is proportional to the number of single-strand breaks produced by the endonuclease.

Cells contain a number of enzymatic mechanisms to repair chemical and physical damage to their DNA[58,98-100]. One mechanism is the excision repair of ultraviolet-induced pyrimidine dimers. This mechanism involves four (perhaps five) general steps: (the action of N-glycosidase), incision, excision, polymerization, and ligation. The effect of temperature on excision repair in mammalian cells was measured by using the endonuclease from M. luteus. An effect of temperature observed with the use of this

technique implies that heat must significantly affect, at least, the incision step involved in excision repair.

MATERIALS AND METHODS

Cells

Chinese hamster lung fibroblasts (V-79) and the normal human skin fibroblasts Rid Mor CRL 1220 were obtained from R. B. Setlow, Biology Department, Brookhaven National Laboratory, Upton, N.Y. These cells were grown under humidified 10% CO_2 in air. The Rid Mor cells were at passages 14 through 21 and were transferred at a 3:1 split ratio.

Normal skin fibroblasts (736 NF) were obtained at passage 18 from J. E. Trosko, Human Development Department, Michigan State University, East Lansing, MI., and were grown under humidified 5% CO_2 in air.

Media

Growing cell cultures used for both stocks and experiments utilized a "D" medium (see Appendix A) supplemented with 5% fetal calf serum for the Chinese hamster cells and 10% fetal calf serum for the human fibroblasts. The confluent cells utilized for the experiments were maintained in "D" medium without arginine or isoleucine (see Appendix A) supplemented with 5% dialysed fetal calf serum for the V-79 cells and 10% dialysed fetal calf serum for both strains of human fibroblasts. All media were supplemented with penicillin (160 units/ml) and streptomycin (160 µg/ml).

Experimental Culture Procedure

Approximately 100,000 to 200,000 cells were inoculated into 60 mm plastic Petri dishes (Corning Glass Works, Corning, N.Y.)

containing 6 ml of "D" medium supplemented with fetal calf serum. The cells were allowed to grow at 37°C for 24 hours or less until they were almost at confluence. At this point, the cells were either labeled with ^{3}H-Thymidine at 0.1 to 0.3 μCi/ml (6.7 Ci/mmol, New England Nuclear, Boston, Mass.) or with ^{14}C-Thymidine at 0.03 μCi/ml (50 mCi/mmol, New England Nuclear, Boston, Mass.) at 37°C. The V-79 cells were labeled for 18 hours, while the human fibroblasts were labeled for 48 hours. After this labeling period, the plates were at heavy confluent densities, containing either 4×10^{6} Chinese hamster cells or 4×10^{5} human fibroblasts. At this point, the medium was changed to a "D" medium deficient in arginine and isoleucine, supplemented with dialysed fetal calf serum at 5% (for V-79 cells) or 10% (for human cells). The cells were maintained in this deficient medium at either 37°C or at 41°C between 24 and 144 hours. After the initial 24 hours of the incubation period, the medium was changed to fresh deficient medium. After this incubation period, the cells were sometimes incubated at 37°C for 1 hour with hydroxyurea added to a final concentration of 5 mM.

Ultraviolet Irradiation and Repair

The medium was decanted from the plates and saved just prior to ultraviolet irradiation. The tops were removed from the dishes and the cells were exposed to 254 nm ultraviolet radiation. The V-79 cells were always irradiated at a dose of 2.5 J/m^{2}, while the human cells were always irradiated at a dose of 20 J/m^{2}. Immediately after ultraviolet irradiation, the cells were incubated at 37°C in the spent, decanted medium for 0, 6, or 24 hours.

Cell Lysis and Phenol Extraction of the DNA

After the post-treatment incubation period, the medium was discarded and the cells were washed twice with either cold phosphate buffered saline (for V-79 cells) or an EDTA-containing saline solution (for human cells). Cell detachment from the plates was facilitated by a rubber policeman in 2.5 ml of the same solution. The cell suspension was transferred to centrifuge tubes. Sometimes suspensions of cells that were labeled with different isotopes were combined at this point. After centrifugation, the tubes were inverted to drain.

The pellet was resuspended in a solution containing 0.1 M Tris-HCl (pH 8), 0.2 M NaCl, and 0.5 M EDTA. Up to 1 ml of this solution was used to resuspend one plate of V-79 cells, while up to 2 ml was used for V-79 cells from two plates containing different radioactive isotopes. For human fibroblasts, 0.15 ml of the solution was used for cells from one plate, while 0.3 ml was used for cells from two plates containing different label. The cells were then pipetted to smaller tubes; 10% Sarkosyl (Ciba Geigy Corporation, Ardsley, N. Y.) in the same solution was added to the cell suspension to a final concentration of 0.33%; pronase (Calbiochem, La Jolla, Calif.) was added to give 0.13 mg/ml, and the cell suspension was incubated at 45°C for 10 minutes to lyse the cells. After this period, the lysed cells were usually frozen at -20°C.

After thawing, the lysed cells were incubated at 37°C for one hour. If necessary, the lysed cells from two tubes each containing cells from only one plate were combined before this incubation

period. An equal amount of phenol, neutralized at pH 8 and equilibrated with endonuclease buffer [0.02 M Tris-HCl (pH 8), 0.04 M NaCl, 1 mM EDTA], was added to the lysate, and the samples were gently rotated for 1 to 2 hours at room temperature. The DNA solution was separated from the phenol by centrifugation at room temperature. The upper aqueous phase containing DNA was collected and extracted twice with an equal volume of ether to remove most of the phenol. The DNA solution was dialysed overnight against 500 ml of endonuclease buffer at 4°C.

The Endonuclease Assay

The endonuclease used was a crude extract equivalent to Fraction III of Carrier and Setlow[101]. Five microliters of the extract (5 mg protein per ml) was added to each 100 µl of the DNA solution. Incubation at 37°C for 20 minutes was sufficient to take the enzymatic reaction to completion. Usually either 100 µl or 200 µl of the DNA was used in the endonuclease assay. The reaction was terminated by removing the mixture with a wide-tipped micropipet and layering it on top of a 5% to 20% alkaline sucrose gradient containing 0.5 M NaCl; with 0.2 ml of a lysing solution, containing 0.5 M NaOH and 0.05 M EDTA layered on its top, and a 60% sucrose cushion added to its bottom. The DNA was sedimented at 20°C in an SW 60 rotor of a Beckman L5-50 ultracentrifuge at 50,000 rpm for 60 to 120 minutes. An SW 56 rotor was used for the DNA from human 736 NF cells. Fractions were collected starting from the bottom of the gradient and the acid-insoluble radioactive material was placed in vials containing a scintillation cocktail of 12 g Permablend (90% PPO and 10% M_2-POPOP) per 3.8 liters toluene and was counted in a scintillation counter.

The labeled DNA from V-79 and Rid Mor cells was counted in either a Beckman or Packard counter in the laboratory of R. B. Setlow, Biology Department, Brookhaven National Laboratory, Upton, N.Y. The labeled DNA from 736 NF human fibroblasts was counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 3320), with discriminators set between 250 and 500 for tritium and between 200 and 1000 for ^{14}C. The gain was set at 100% for tritium and 6.85% for ^{14}C.

The distribution of counts was converted to average molecular weights by a computer program as described elsewhere[72] and in Appendix B. The amounts of radioactivity per gradient were between 3,000 and 12,000 cpm of tritium and between 2,000 and 7,000 cpm of ^{14}C from V-79. There was between 800 and 8,000 cpm of each isotope in DNA from human cells in each gradient.

Calculations of Endonuclease-Sensitive Sites

The weight-average molecular weight,

$$M_w = \frac{\Sigma m_i n_i \cdot m_i}{\Sigma m_i n_i} ,$$

was used rather than the number-average molecular weight,

$$M_n = \frac{\Sigma m_i n_i}{\Sigma n_i} ,$$

because M_n is very sensitive to fluctuations in the amount of DNA near the top of the gradient. It was assumed that the breaks were distributed randomly and therefore $M_n = M_w/2$. The number of breaks per unit weight of DNA is the reciprocal of M_n. The change in $1/M_n$ as a result of endoclease treatment should represent the number of endonuclease-sensitive sites per dalton of DNA.

RESULTS

Experiments Involving Chinese Hamster Lung Fibroblasts

The use of M. luteus endonuclease provides a sensitive means to detect pyrimidine dimers in DNA induced by ultraviolet radiation. The DNA from Chinese hamster lung (V-79) cells was always incubated at confluence at either 37°C or 41°C for 72 hours before ultraviolet irradiation in a medium deficient in arginine and isoleucine. After this 72 hour period, the cells were incubated for 1 hour at 37°C with hydroxyurea, added at a concentration of 5 mM. The DNA from the V-79 cells was always sedimented at 50,000 rpm for 60 minutes.

In one experiment, V-79 cells were incubated for 3 days at 37°C and then were either exposed to 2.5 J/m^2 UV radiation or not exposed. After irradiation, the UV irradiated cells were mixed and lysed immediately with non-irradiated cells. The resulting sedimentation data of Figure 24 indicates that the M_w for UV irradiated cells is 23.49×10^6 daltons and for non-irradiated cells is 64.43×10^6 daltons. The number of endonuclease sites per dalton that were produced by 2.5 J/m^2 UV radiation is given by $2(1/23.49 - 1/64.43) \times 10^{-6} = 5.4 \times 10^{-8}$. When the same experiment was repeated without using the endonuclease, the M_w for UV irradiated cells was 61.75×10^6 daltons and for non-irradiated cells was 62.48×10^6 daltons. The number of breaks per dalton that were produced by 2.5 J/m^2 UV irradiation was $2(1/61.75 - 1/62.48) \times 10^{-6} = 0.4 \times 10^{-8}$. Thus, ultraviolet radiation alone produces an insignificant number of breaks in DNA when this is compared to the number of endonuclease sensitive sites it produces.

Figure 24. Sedimentation profiles of extracted DNA from V-79 cells after treatment with M. luteus endonuclease. Values of M_w were 64.43 x 10^6 (Fractions 1 to 27) for non-irradiated cells (solid circles) and 23.49 x 10^6 (Fractions 5 to 28) for cells ultraviolet irradiated at 2.5 J/m^2 (hollow circles)

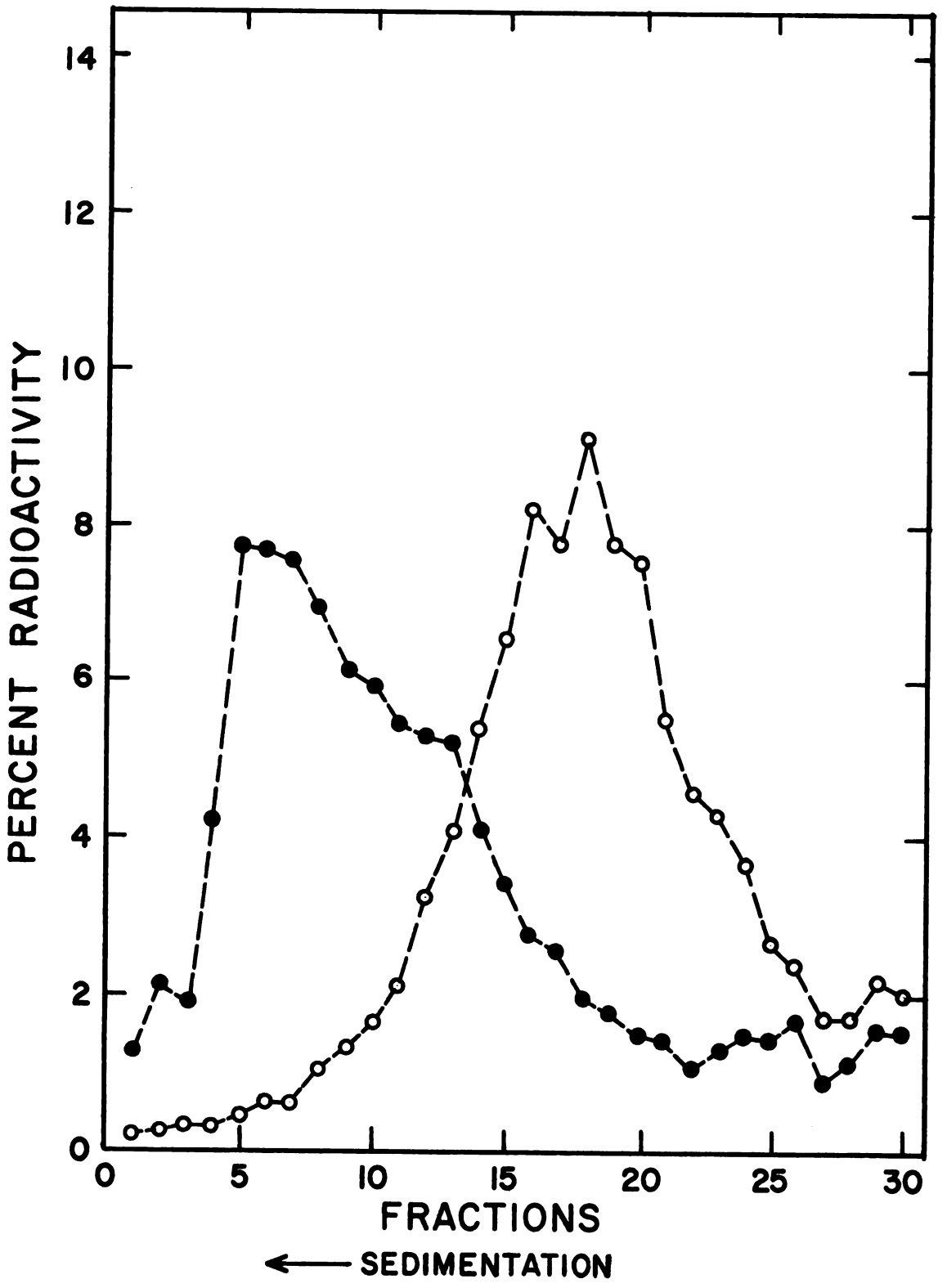

PERCENT RADIOACTIVITY
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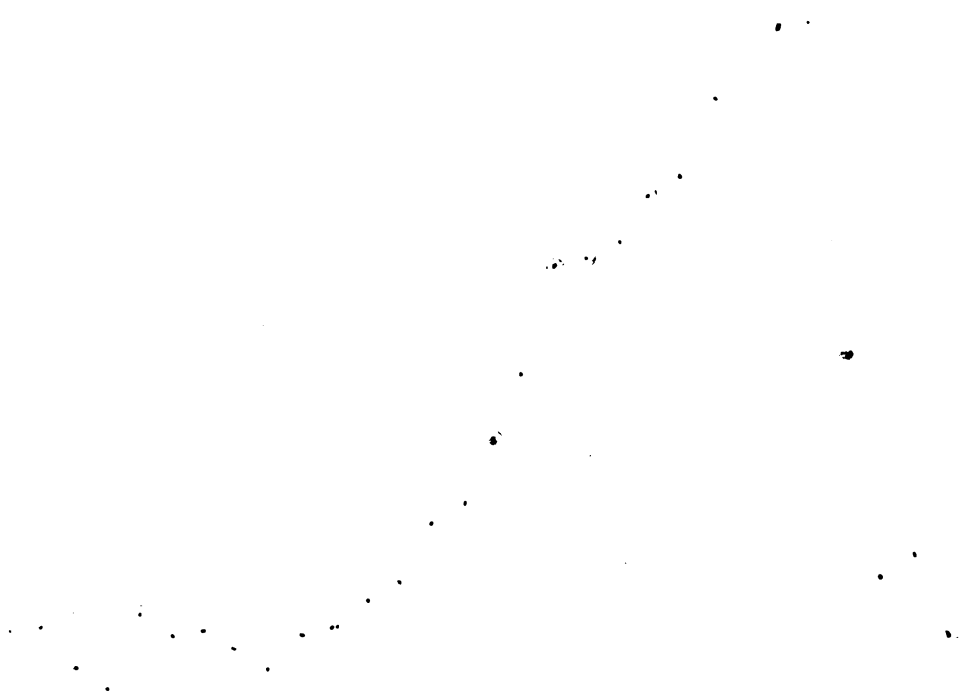
Figure 24.



In another experiment, the V-79 cells were incubated for 3 days at 37°C, and subsequently exposed to 2.5 J/m^2 ultraviolet radiation. After UV irradiation, the cells were either lysed immediately for 10 minutes at 45°C, and then frozen, or were incubated for 6 hours at 37°C before being lysed and frozen. After thawing, the lysed cells were mixed and the remainder of the procedure was carried out. The resulting sedimentation data of Figure 25 indicates that cells lysed immediately after UV irradiation had M_w of 20.77×10^6 daltons and that cells that were incubated for 6 hours at 37°C, after UV irradiation, had M_w of 26.50×10^6 daltons. The number of endonuclease sites per dalton that were removed during the 6 hour incubation period is $2(1/20.77 - 1/26.50) \times 10^{-6} = 2.1 \times 10^{-8}$.

In order to determine the effect of temperature on the amount of endonuclease sites removed, the cells were incubated at either 37°C or 41°C for 3 days before UV irradiation. After UV treatment at 2.5 J/m^2, the cells were incubated at 37°C for 6 hours. After this 6 hour incubation period, the cells were mixed together and the rest of the procedure was carried out. The resulting sedimentation data of Figure 26 indicates that M_w is 23.18×10^6 daltons for cells incubated for 3 days at 41°C and 25.37×10^6 daltons for cells incubated for 3 days at 37°C. The difference in the number of endonuclease sites removed per dalton during a 6 hour incubation is $2(1/23.18 - 1/25.37) \times 10^{-6} = 0.74 \times 10^{-8}$. In an identical experiment, this difference was $2(1/25.53 - 1/28.85) \times 10^{-6} = 0.90 \times 10^{-8}$. Another identical experiment conducted without endonuclease, but with the same samples as in the experiment just mentioned, gave the difference $2(51.25 - 1/53.28) \times 10^{-6} = 0.15 \times 10^{-8}$. Therefore the

Figure 25. Sedimentation profiles of extracted DNA from V-79 cells after treatment with M. luteus endonuclease. Cells were exposed to 2.5 J/m^2 ultraviolet radiation. Values of M_w were 20.77 x 10^6 (Fractions 2 to 26) for DNA extracted immediately after irradiation (solid circles) and 26.50 x 10^6 (Fractions 2 to 26) for DNA extracted 6 hours after irradiation (hollow circles)

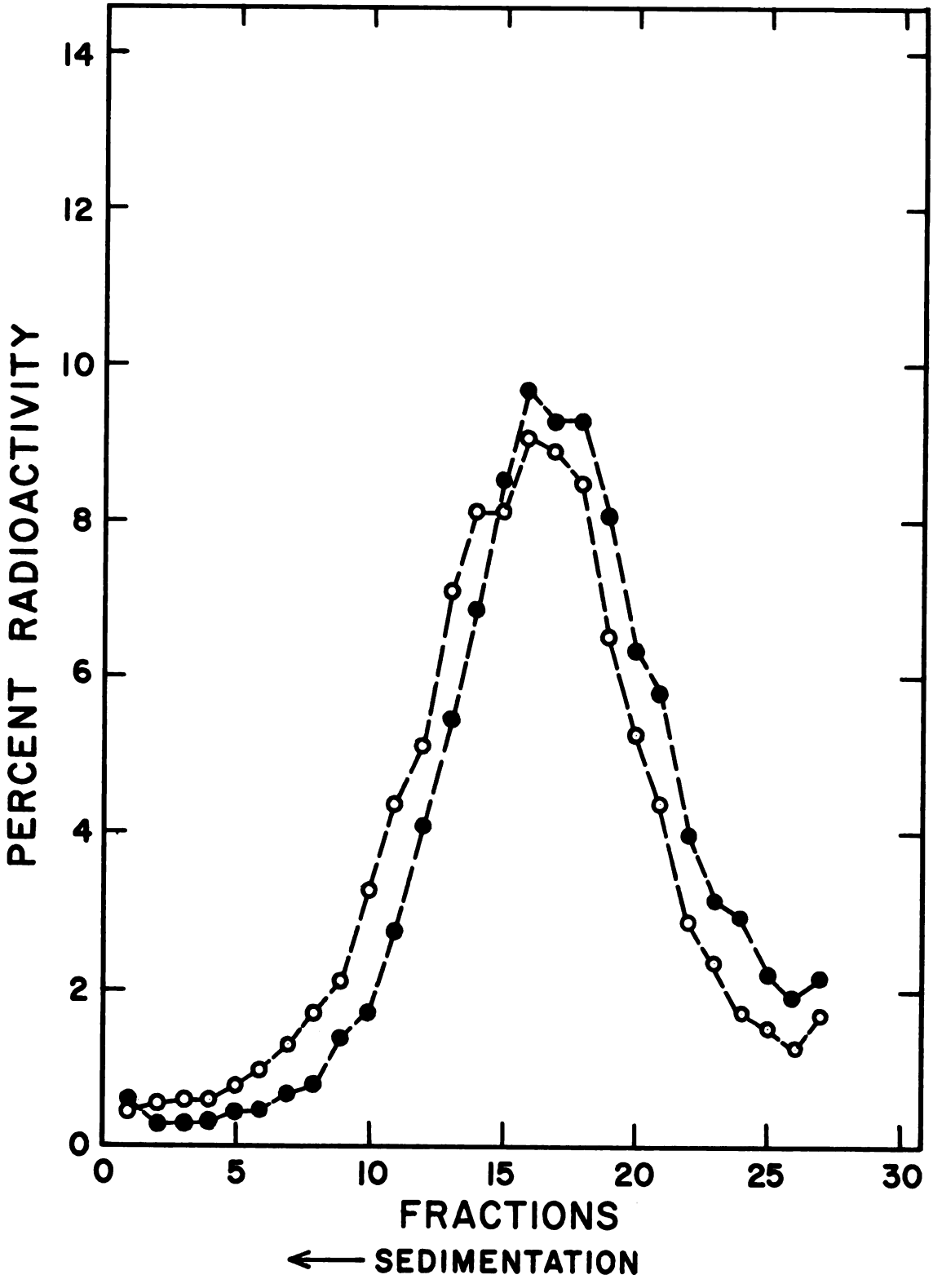

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Figure 25.

Figure 26. Sedimentation profiles of extracted DNA from V-79 cells after treatment with M. luteus endonuclease. Cells were exposed to 2.5 J/m^2 ultraviolet radiation and the DNA was extracted 6 hours after irradiation. Values of M_w were 25.37 x 10^6 (Fractions 1 to 25) for cells that were incubated at 37°C for 72 hours before irradiation (solid circles) and 23.18 x 10^6 (Fractions 1 to 25) for cells that were incubated at 41°C for 72 hours before irradiation (hollow circles)

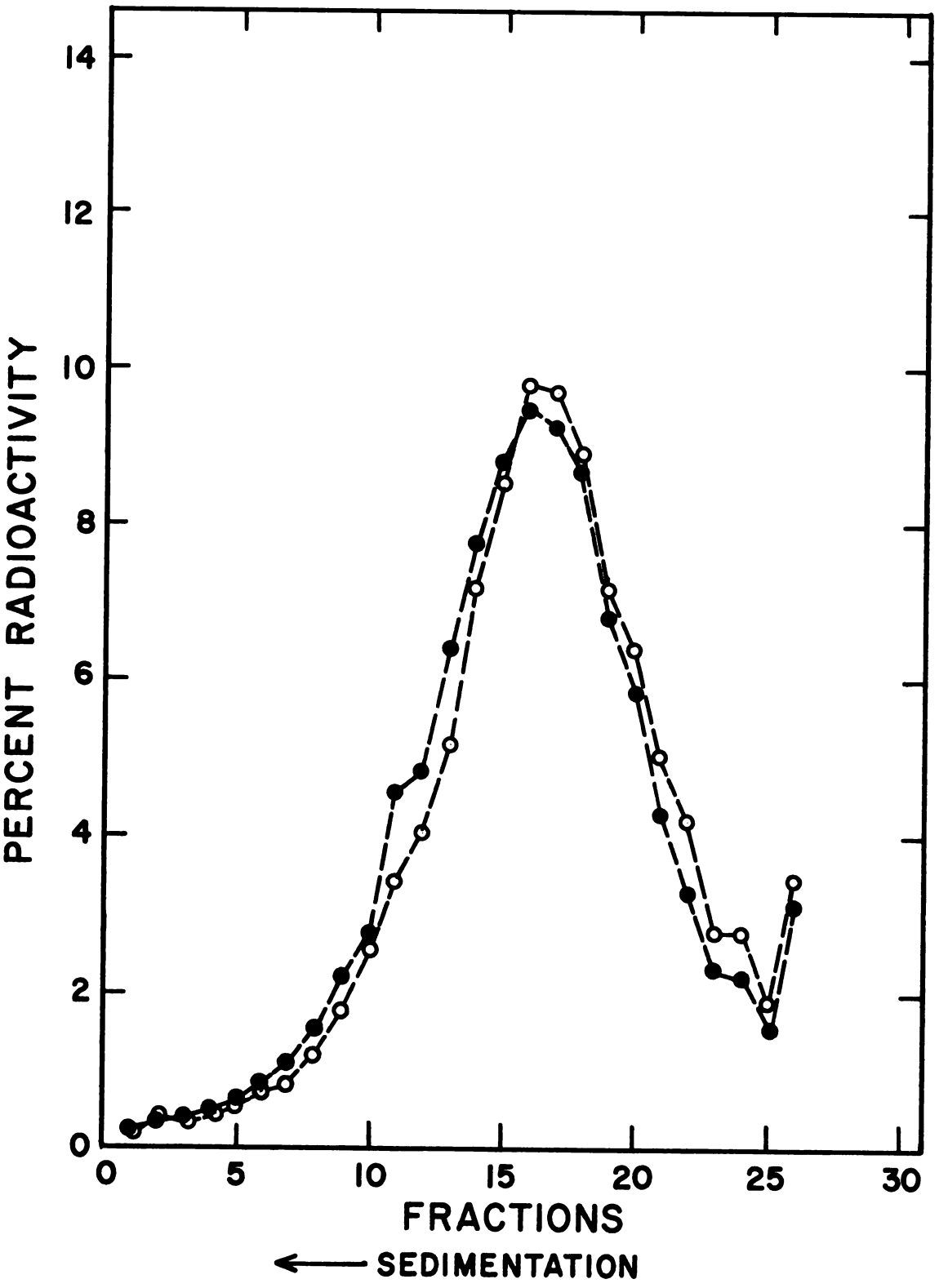
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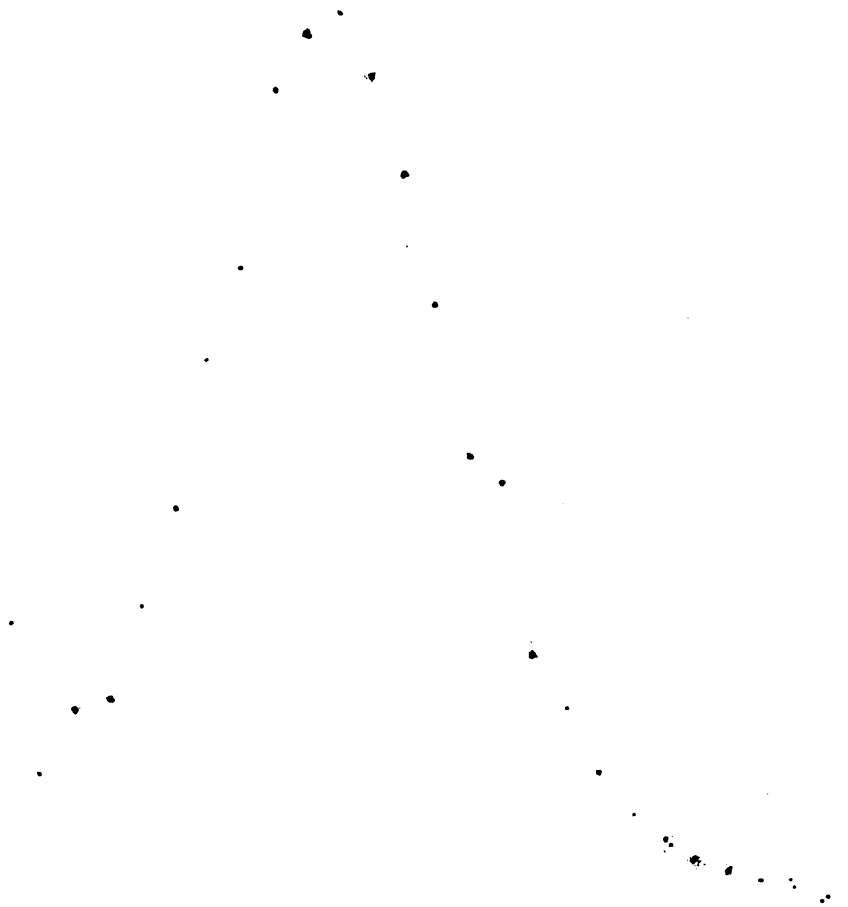
Figure 26.



difference in the removal of endonuclease sites between cells incubated for 3 days at 37°C and ones incubated for 3 days at 41°C, per dalton, is $(0.90 - 0.15) \times 10^{-8} = 0.75 \times 10^{-8}$. Thus, in two experiments, pre-UV hyperthermia results in the inhibition of subsequent removal of endonuclease sensitive sites that are produced by ultraviolet radiation.

Experiments Involving Human Skin Fibroblasts

When human skin fibroblasts were used, the pre-UV incubation period was varied between 1 and 6 days, while the cells were maintained at confluence in a medium deficient in arginine and isoleucine. After this period, hydroxyurea was either added at a concentration of 5 mM for a period of one hour at 37°C, or not used. Sedimentation of DNA was always at 50,000 rpm, but the period varied from 60 to 120 minutes.

Figure 27 shows the sedimentation data for the Rid Mor cells that were incubated for 3 days at 37°C and treated with hydroxyurea before they were either exposed to 20 J/m^2 of ultraviolet radiation or were not irradiated. The number of breaks produced per dalton by UV radiation and endonuclease action was $2(1/7.06 - 1/51.75) \times 10^{-6} = 24.5 \times 10^{-8}$. When the identical experiment was run with the same samples, but with the endonuclease left out, the number of breaks per dalton produced by the UV irradiation was $2(1/45.32 - 1/47.15) \times 10^{-6} = 0.17 \times 10^{-8}$. Therefore, the number of endonuclease sites produced per dalton by UV radiation was $(24.5 - 0.17) \times 10^{-8} = 24.3 \times 10^{-8}$.

In another experiment, the Rid Mor cells were incubated for 3 days at 37°C and treated with hydroxyurea before UV irradiation

Figure 27. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Values of M_w were 51.75 x 10^6 (Fractions 1 to 29) for non-irradiated cells (solid circles) and 7.06 x 10^6 (Fractions 14 to 29) for cells ultraviolet irradiated at 20 J/m^2 (hollow circles)

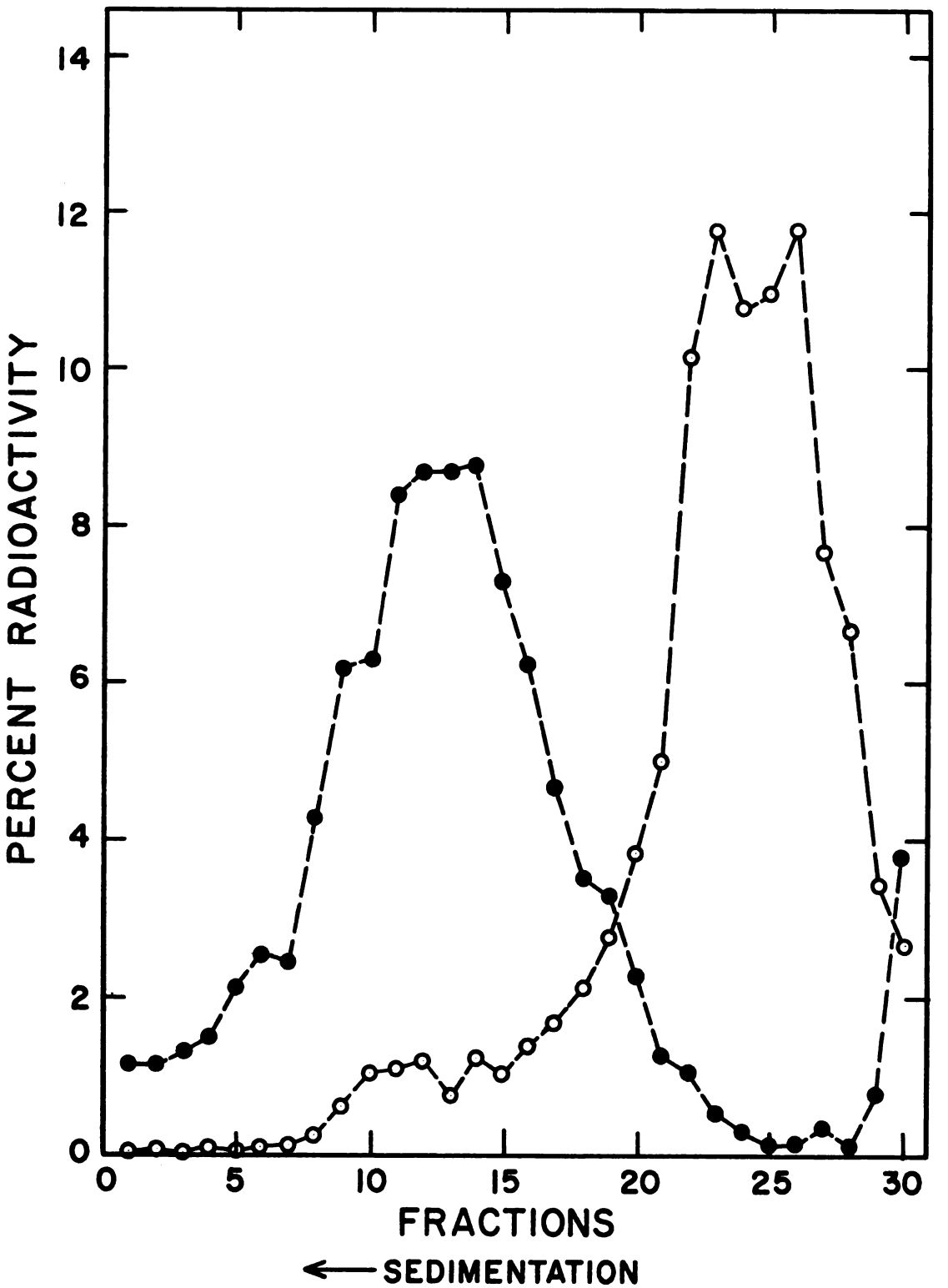

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Figure 27.

at 20 J/m^2. After irradiation, the cells were either lysed immediately and then frozen, or were incubated for 24 hours at 37°C before being lysed and frozen. The cells were then thawed and mixed together before the remainder of the procedure was carried out. The 80 minute sedimentation data of Figure 28 indicates that the number of endonuclease sites removed per dalton by the 24 hour post-UV incubation period was $2(1/5.77 - 1/7.74) \times 10^{-6} = 8.8 \times 10^{-8}$.

Figure 29 shows the 80 minute sedimentation data for Rid Mor cells incubated for 3 days at either 37°C or 41°C, and treated with hydroxyurea, before being UV irradiated at 20 J/m^2. After irradiation, the cells were incubated for 24 hours at 37°C before being mixed together. The difference between the number of breaks per dalton in the DNA from cells incubated at 41°C and cells incubated at 37°C was $2(1/7.66 - 1/8.06) \times 10^{-6} = 1.3 \times 10^{-8}$. The identical samples were run in the same manner except that their DNA was not treated with endonuclease and was sedimented for 60 minutes. The difference between the number of breaks per dalton from cells incubated at 41°C and cells incubated at 37°C was $2(1/68.85 - 1/71.88) \times 10^{-6} = 0.12 \times 10^{-8}$. Therefore the difference between the number of endonuclease sites removed per dalton, in the 24 hour incubation period, between cells that were incubated at 37°C and those incubated at 41°C, in the three day pre-UV period, was $(1.3 - 0.12) \times 10^{-8} = 1.2 \times 10^{-8}$. Thus pre-UV hyperthermia inhibited the subsequent removal of endonuclease sites.

Rid Mor cells, in another experiment, were incubated for 3 days at either 37°C or 41°C and were treated with hydroxyurea. After UV irradiation at 20 J/m^2, the cells were mixed immediately. DNA was

Figure 28. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Cells were exposed to 20 J/m^2 ultraviolet irradiation. Values of M_w were 5.77×10^6 (Fractions 8 to 25) for DNA extracted immediately after irradiation (solid circles) and 7.74×10^6 (Fractions 8 to 25) for DNA extracted 24 hours after irradiation (hollow circles)

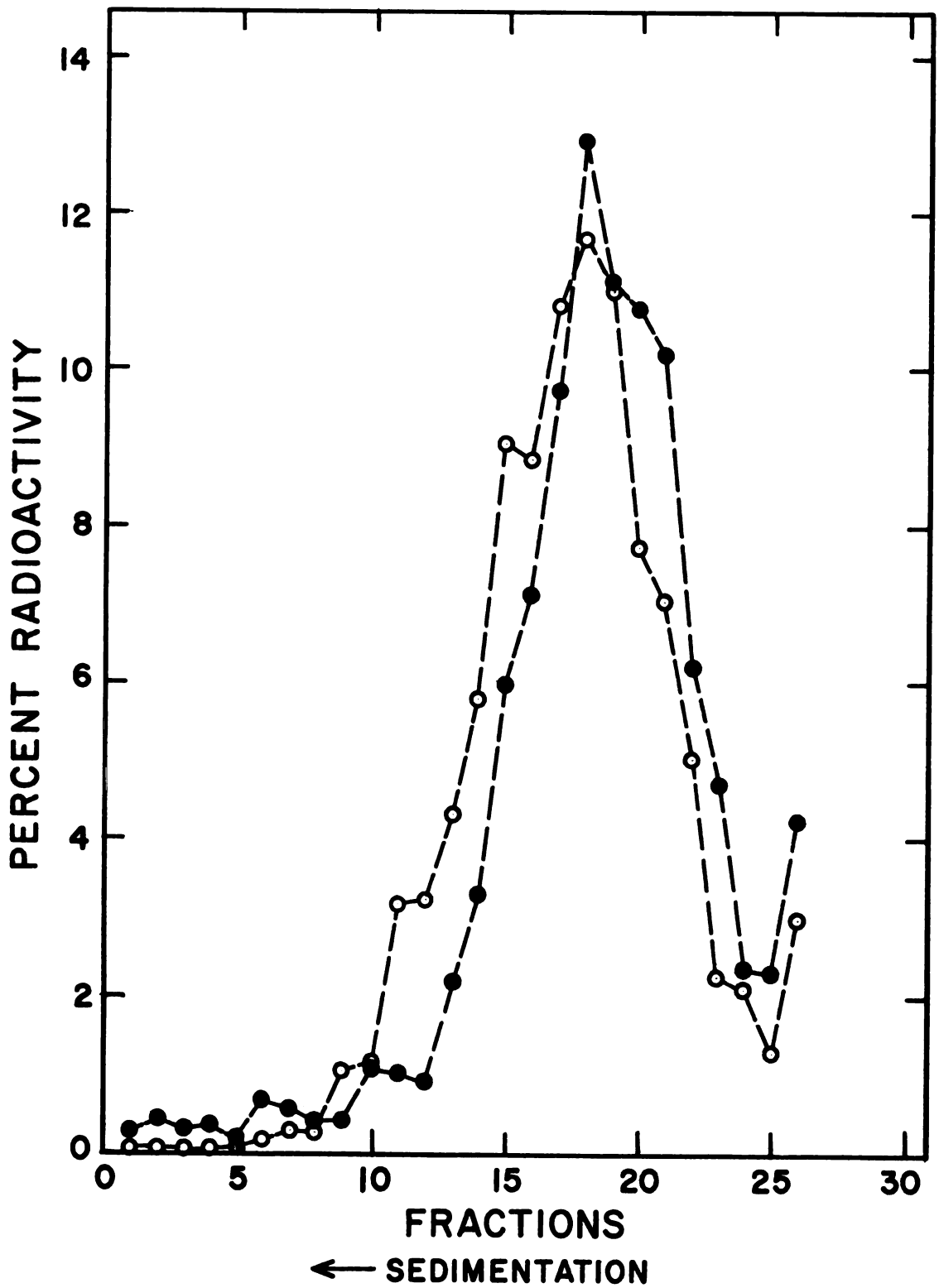
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Figure 28.

Figure 29. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Cells were exposed to 20 J/m^2 ultraviolet radiation and the DNA was extracted 24 hours after irradiation. Values of M_w were 8.06×10^6 (Fractions 5 to 25) for cells that were incubated at 37°C for 72 hours before irradiation (solid circles) and 7.66×10^6 (Fractions 5 to 25) for cells that were incubated at 41°C for 72 hours before irradiation (hollow circles)

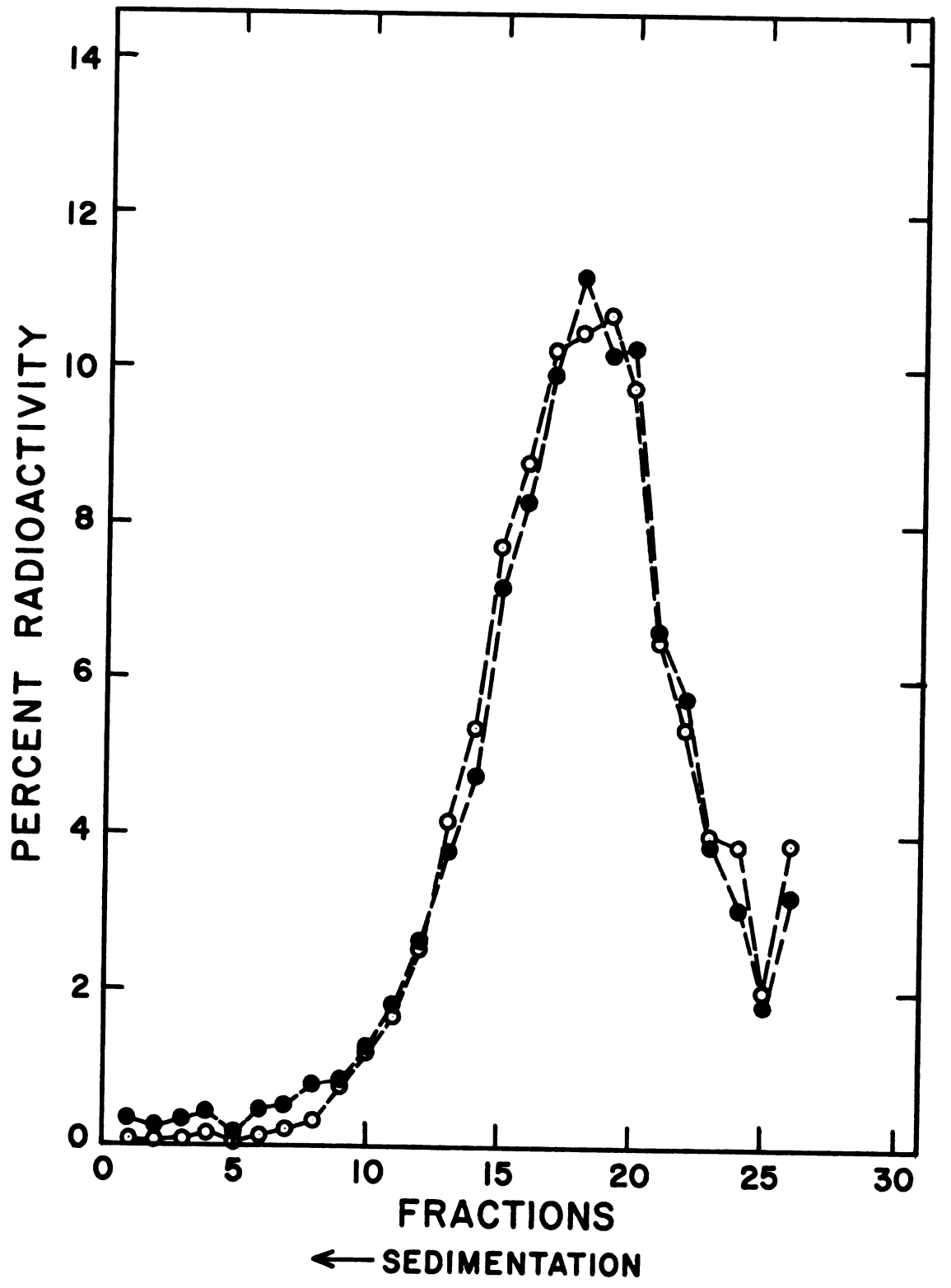
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Figure 29.

extracted and sedimented for 120 minutes. The endonuclease from M. luteus was not used. The difference in the number of breaks per dalton in the DNA of cells incubated at 37°C and those incubated at 41°C was $2(1/4.01 - 1/4.04) \times 10^{-6} = 0.4 \times 10^{-8}$. Thus pre-UV hyperthermia did not enhance the amount of breaks in UV-irradiated DNA.

Hydroxyurea was not added in the following experiment to determine if its presence affected the number of endonuclease sensitive sites. Also the period of pre-UV hyperthermia was varied to determine if the length of this period would affect subsequent removal of endonuclease sites. Human cells were incubated for 1, 3, or 6 days, at either 37°C or 41°C, in medium deficient in arginine and isoleucine. Following this period, cells with identical treatments, but labeled with different radioisotopes, were either UV irradiated with 20 J/m^2 and then incubated for 24 hours at 37°C, or were first incubated at 37°C for 24 hours before UV irradiation at 20 J/m^2. The cells, with differently labeled DNA, were immediately mixed together and lysed after this treatment. The 120 minute sedimentation data for Rid Mor cells is shown in Figures 30 to 35. The number of endonuclease sites removed per dalton in 24 hours at 37°C is given in the first six rows of Table 2. More endonuclease sites were removed in this experiment than in previously mentioned equivalent experiments that utilized hydroxyurea. The results also show that 6 day pre-UV hyperthermia inhibits removal of endonuclease sites more than 1 or 3 day pre-UV hyperthermia. Extended pre-UV incubation at 37°C did not significantly alter the amount of endonuclease site removal after irradiation.

Figure 30. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Confluent cells were incubated at 37°C for 24 hours in medium deficient in arginine and isoleucine before exposure to 20 J/m^2 ultraviolet radiation. Values of M_w were 3.78×10^6 (Fractions 7 to 32) for DNA extracted immediately after irradiation (solid circles) and 7.96×10^6 (Fractions 1 to 32) for DNA extracted 24 hours after irradiation (hollow circles)

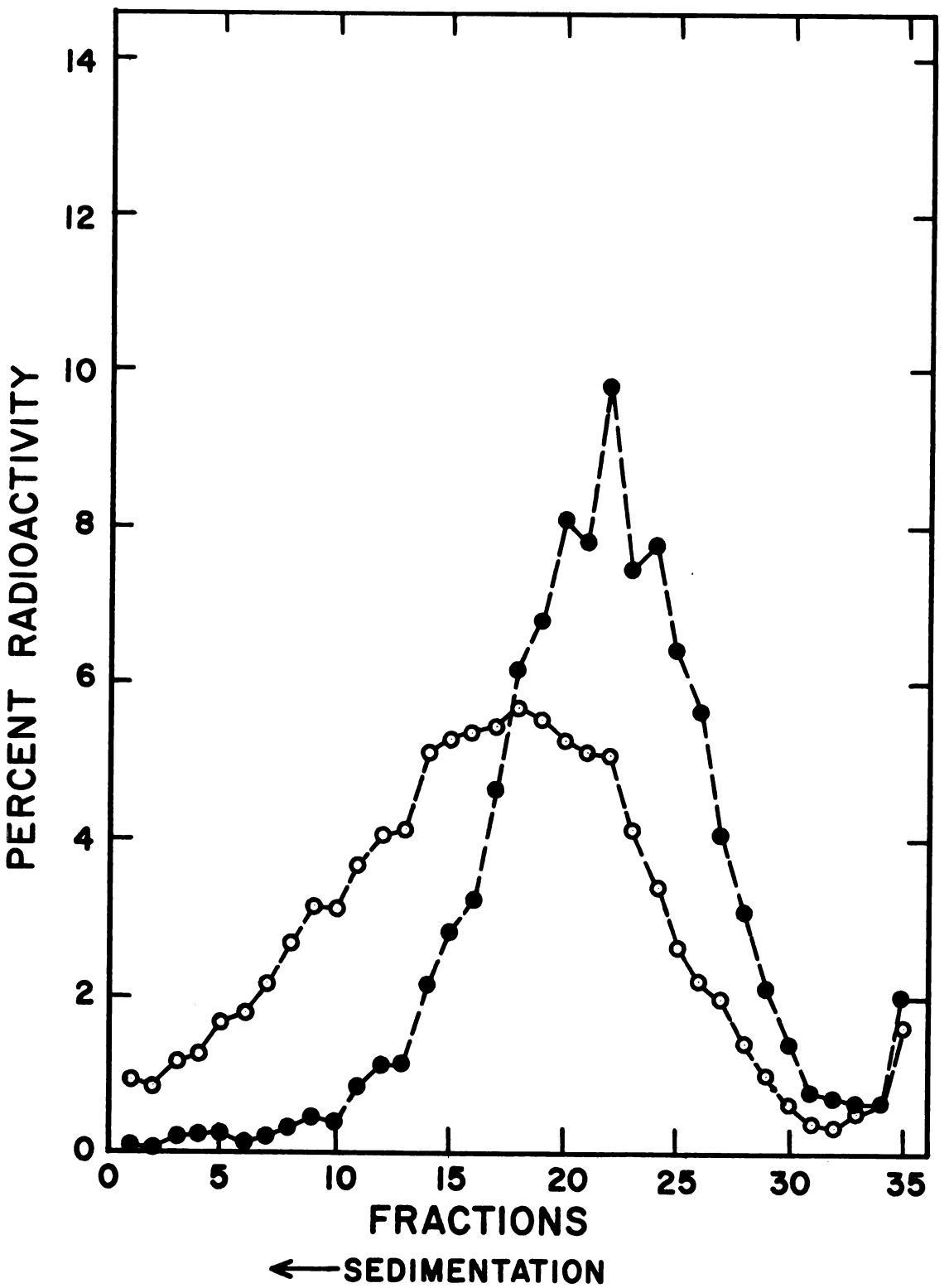
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Figure 30.

Figure 31. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Confluent cells were incubated at 37°C for 72 hours in medium deficient in arginine and isoleucine before exposure to 20 J/m^2 ultraviolet radiation. Values of M_w were 4.05 x 10^6 (Fractions 7 to 31) for DNA extracted immediately after irradiation (solid circles) and 7.88 x 10^6 (Fractions 1 to 30) for DNA extracted 24 hours after irradiation (hollow circles)

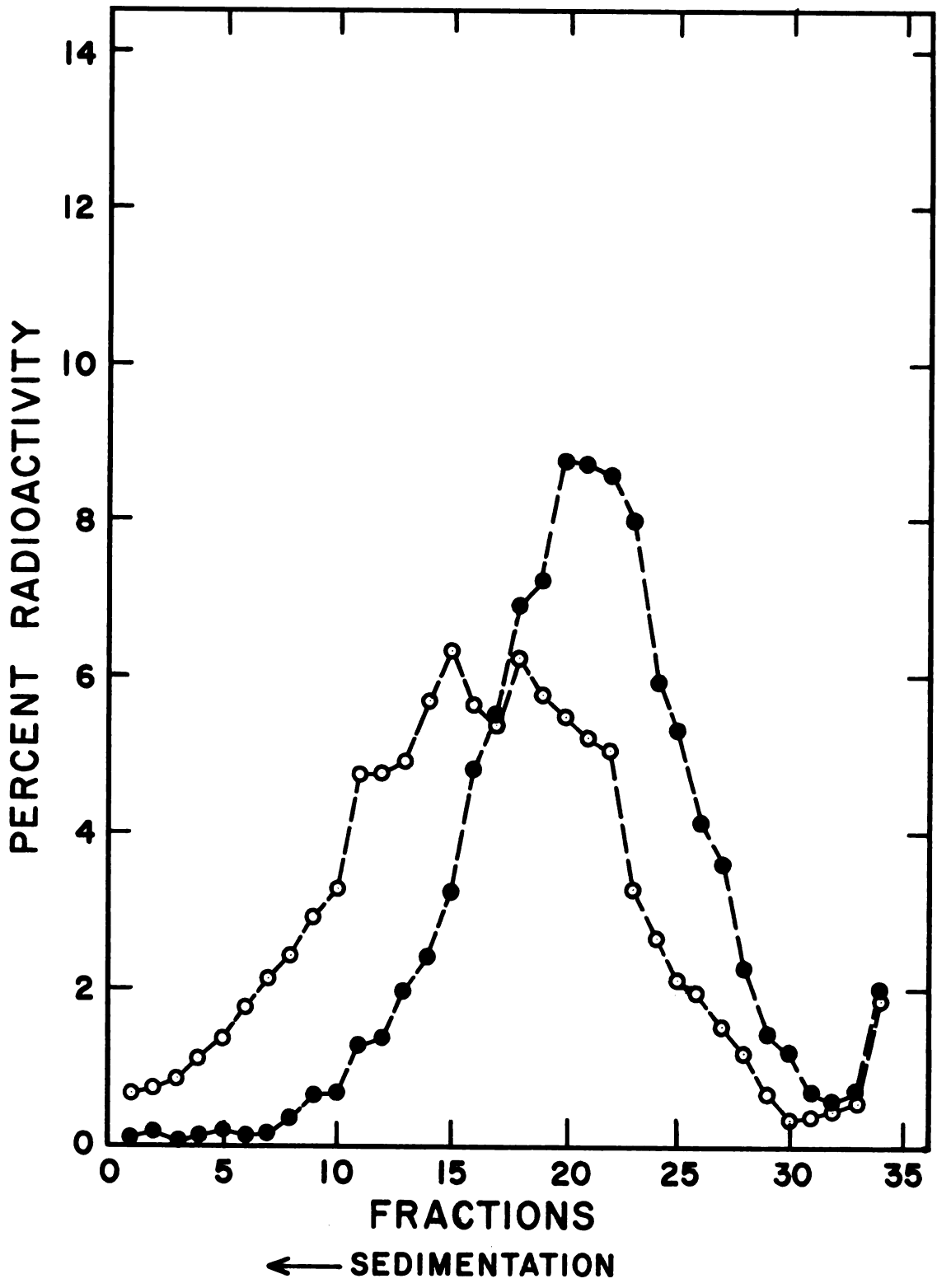
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Figure 31.

Figure 32. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Confluent cells were incubated at 37°C for 144 hours in medium deficient in arginine and isoleucine before exposure to 20 J/m^2 ultraviolet radiation. Values of M_w were 4.04×10^6 (Fractions 8 to 31) for DNA extracted immediately after irradiation (solid circles) and 7.98×10^6 (Fractions 1 to 32) for DNA extracted 24 hours after irradiation (hollow circles)

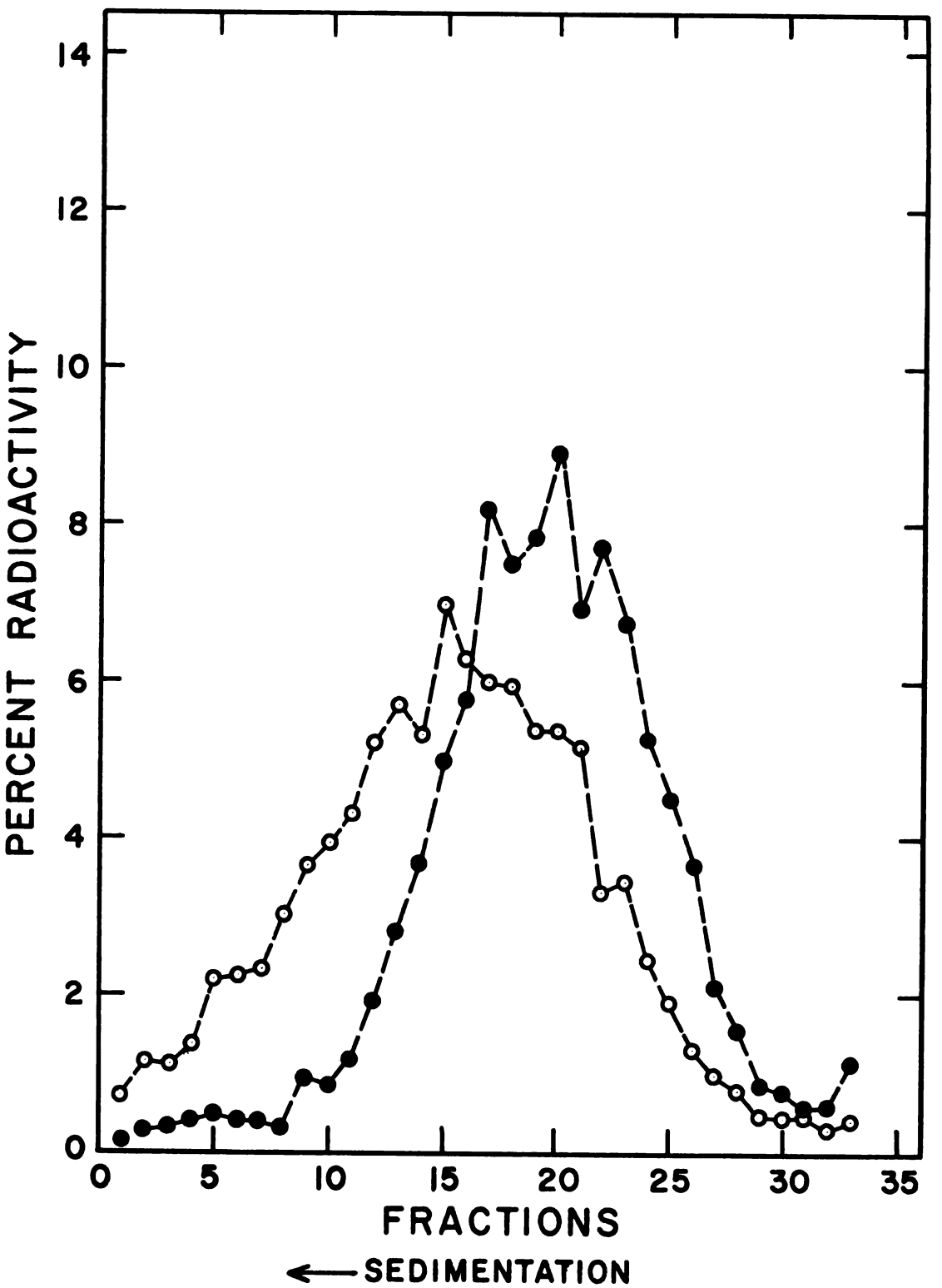
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Figure 32.

Figure 33. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Confluent cells were incubated at 41°C for 24 hours in medium deficient in arginine and isoleucine before exposure to 20 J/m^2 ultraviolet radiation. Values of M_w were 4.17×10^6 (Fractions 9 to 32) for DNA extracted immediately after irradiation (solid circles) and 7.31×10^6 (Fractions 1 to 29) for DNA extracted 24 hours after irradiation (hollow circles)

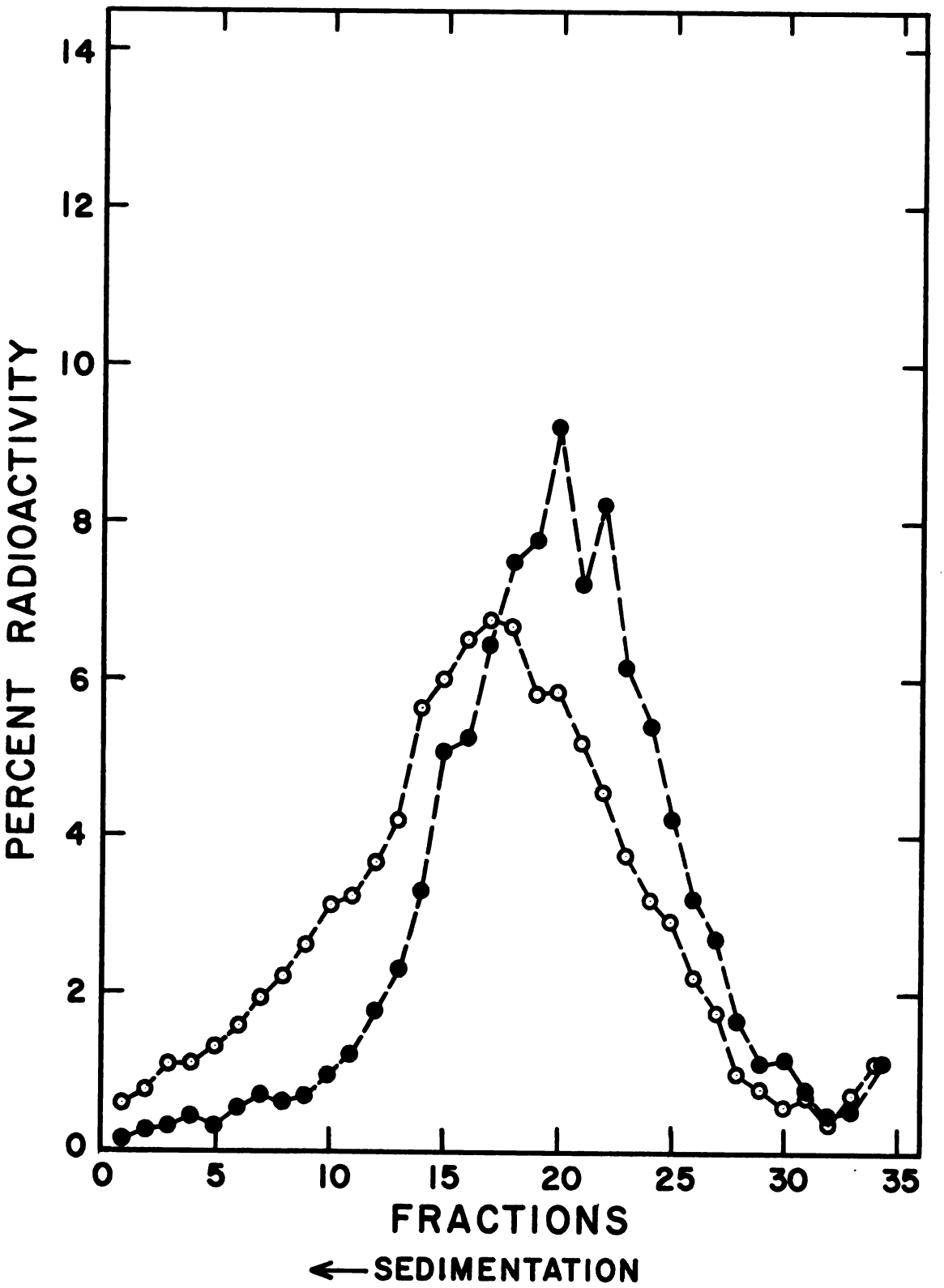
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Figure 33.

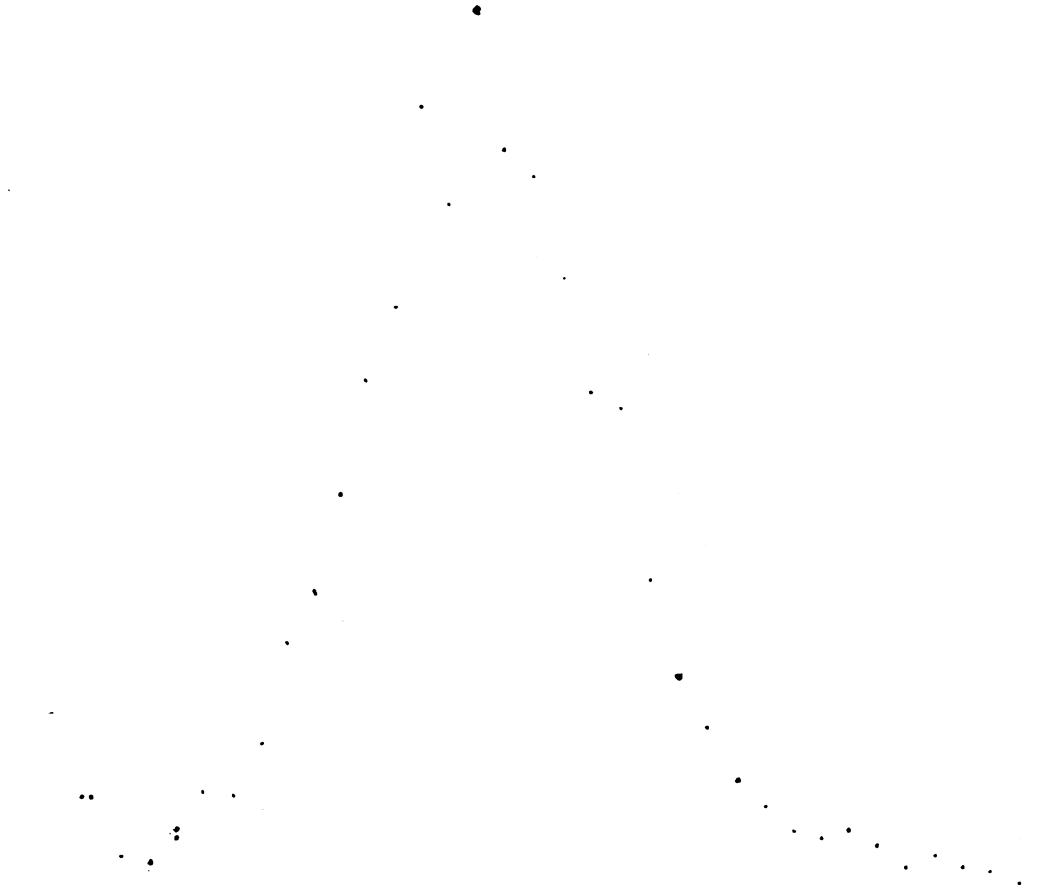

Figure 34. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Confluent cells were incubated at 41°C for 72 hours in medium deficient in arginine and isoleucine before exposure to 20 J/m^2 ultraviolet radiation. Values of M_w were 4.01×10^6 (Fractions 1 to 31) for DNA extracted immediately after irradiation (solid circles) and 6.84×10^6 (Fractions 1 to 31) for DNA extracted 24 hours after irradiation (hollow circles)

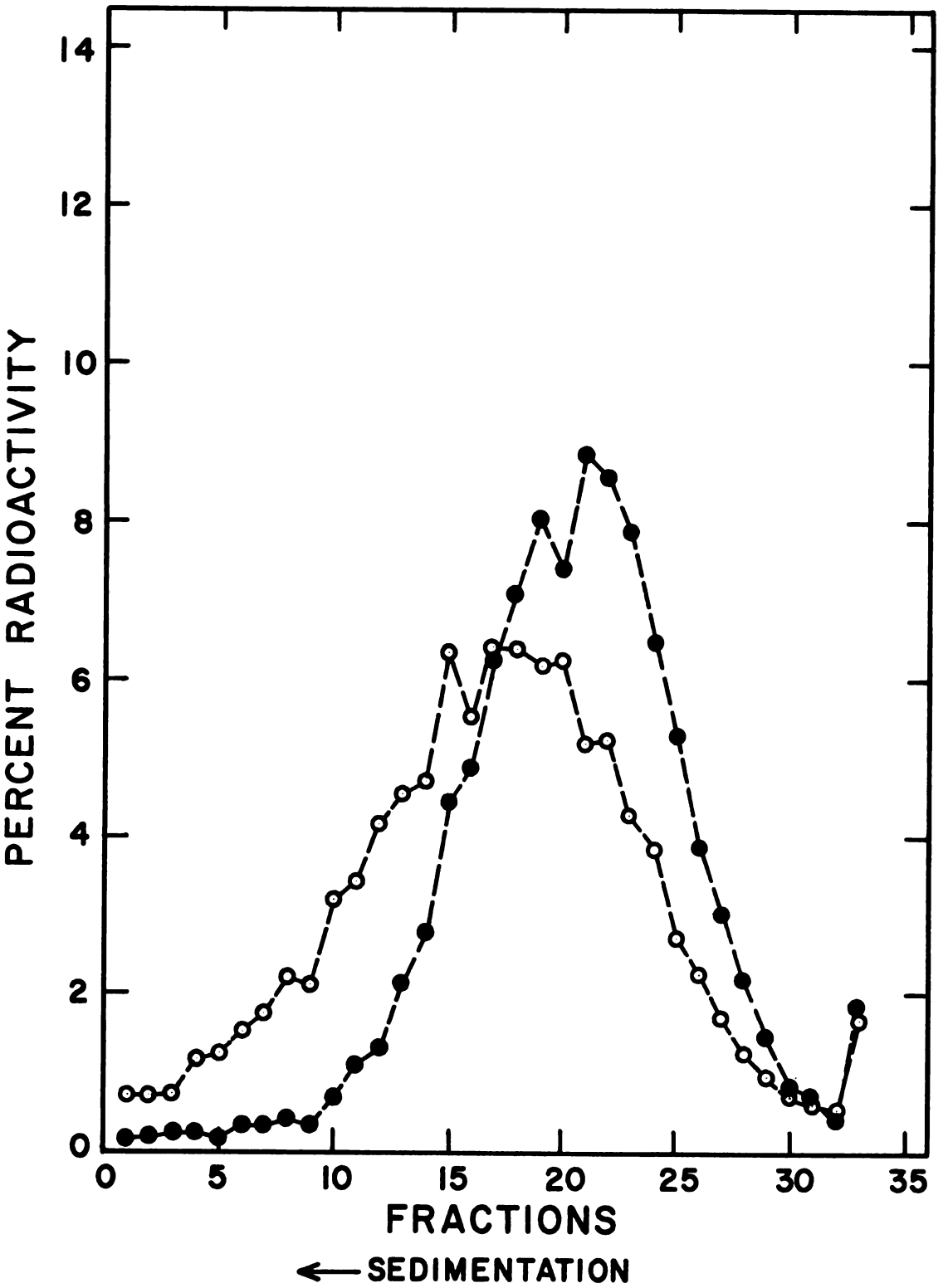
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Figure 34.

Figure 35. Sedimentation profiles of extracted DNA from human skin fibroblasts (Rid Mor) after treatment with M. luteus endonuclease. Confluent cells were incubated at 41°C for 144 hours in medium deficient in arginine and isoleucine before exposure to 20 J/m^2 ultraviolet radiation. Values of M_w were 4.04×10^6 (Fractions 5 to 31) for DNA extracted immediately after irradiation (solid circles) and 5.20×10^6 (Fractions 2 to 31) for DNA extracted 24 hours after irradiation (hollow circles)

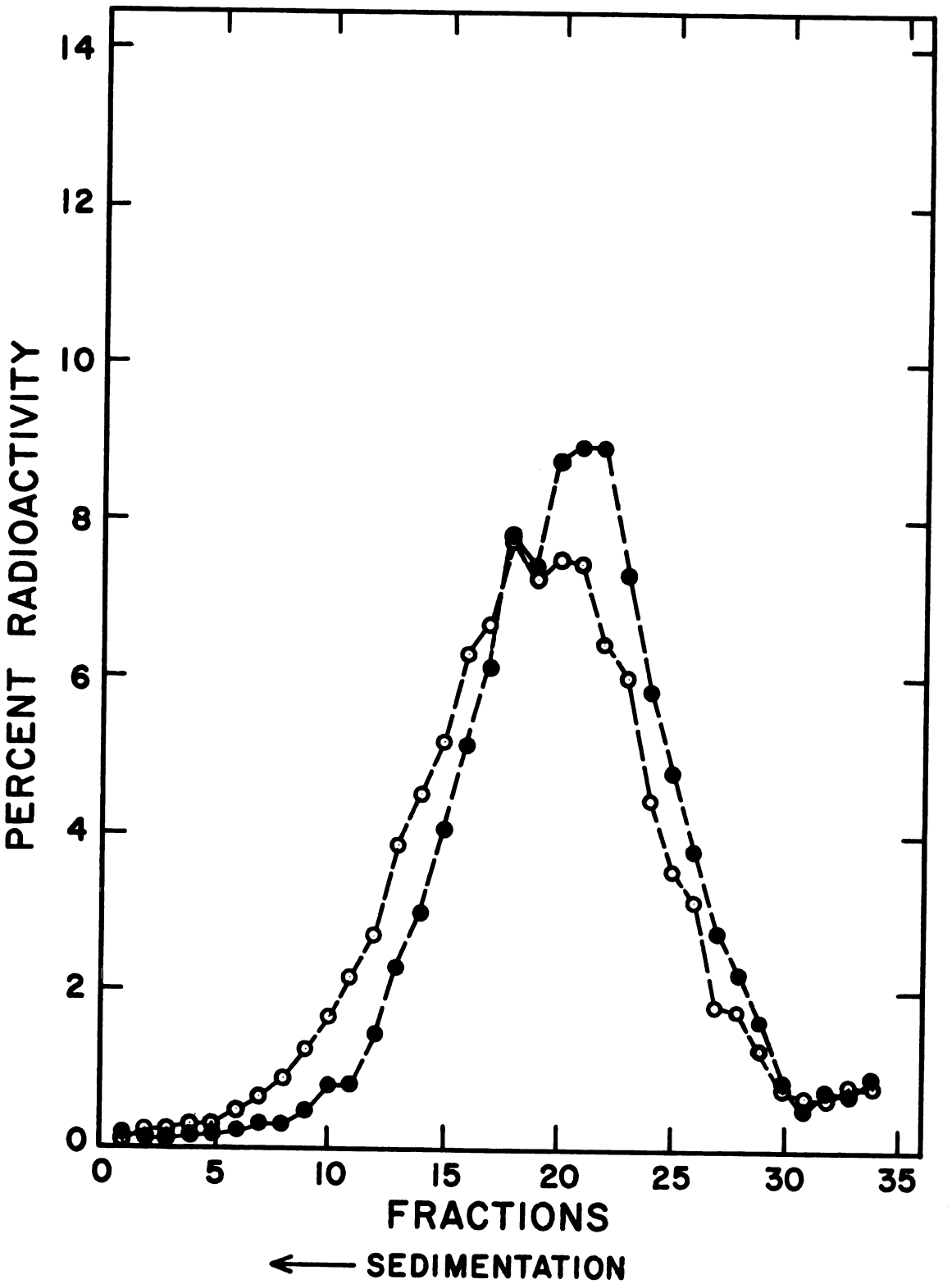
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Figure 35.

Table 2. Endonuclease sensitive sites removed in human skin fibroblasts

Figure in text	Cell type	Initial incubation period before ultraviolet irradiation		Weight average molecular weight, M_w (10^6 daltons)		Number of endonuclease sites removed per 10^8 daltons in 24 hours
		temperature (°C)	time (days)	extraction immediately after ultraviolet irradiation	extraction 24 hours after ultraviolet irradiation	
30	Rid Mor	37	1	3.78	7.96	27.8
31	Rid Mor	37	3	4.05	7.88	24.0
32	Rid Mor	37	6	4.04	7.98	24.4
33	Rid Mor	41	1	4.17	7.31	20.6
34	Rid Mor	41	3	4.01	6.84	20.6
35	Rid Mor	41	6	4.04	5.20	11.0
36	736-NF	41	1	4.04	7.12	21.5
37	736-NF	41	3	5.55	7.91	10.7

When the above experiment was repeated with 736-NF human fibroblasts, pre-UV incubation at 41°C for 6 days resulted in cell detachment from the plates. The 105 minute sedimentation data for 736-NF cells, pre-UV incubated at 41°C for 1 and 3 days, are shown in Figures 36 and 37. The number of endonuclease sites removed per dalton in 24 hours at 37°C is given in the last two rows of Table 2. Increasing the pre-UV period of hyperthermia from 24 hours to 72 hours resulted in a decreased removal of sites after UV irradiation.

A final experiment determined the influence of hydroxyurea on the number of endonuclease sensitive sites. Rid Mor cells, incubated for 3 days at 37°C or 41°C, were either untreated or treated with hydroxyurea at 5 mM for one hour at 37°C before UV irradiation at 20 J/m^2. All DNA was sedimented for 120 minutes. When cells were pre-UV incubated at 37°C, the number of endonuclease sensitive sites per dalton that the untreated cells had over the hydroxyurea treated cells was $2(1/4.20 - 1/4.21) \times 10^{-6} = 0.1 \times 10^{-8}$. When cells were pre-UV incubated at 41°C, this value was $2(1/5.26 - 1/5.18) = 0.6 \times 10^{-8}$. When cells that were pre-UV incubated for 3 days at 37°C were post-UV incubated for 24 hours at 37°C in the same pre-UV medium, the difference in the number of endonuclease sites per dalton between untreated cells and hydroxyurea treated cells was $2(1/7.87 - 1/5.11) \times 10^{-6} = 13.7 \times 10^{-8}$. In a repeated experiment, this value was $2(1/9.42 - 1/6.15) \times 10^{-6} = 11.3 \times 10^{-8}$. This averages to 12.5×10^{-8}. The inhibition that hydroxyurea has on the number of endonuclease sites removed per dalton during 24 hours after UV irradiation is $(12.5 + 0.1) \times 10^{-8} = 12.6 \times 10^{-8}$. Thus hydroxyurea inhibits the removal, but not the production, of endonuclease sensitive sites in DNA.

Figure 36. Sedimentation profiles of extracted DNA from human skin fibroblasts (735-NF) after treatment with M. luteus endonuclease. Confluent cells were incubated at 41°C for 24 hours in medium deficient in arginine and isoleucine before exposure to 20 J/m^2 ultraviolet radiation. Values of M_w were 4.04×10^6 (Fractions 9 to 25) for DNA extracted immediately after irradiation (solid circles) and 7.12×10^6 (Fractions 1 to 25) for DNA extracted 24 hours after irradiation (hollow circles)

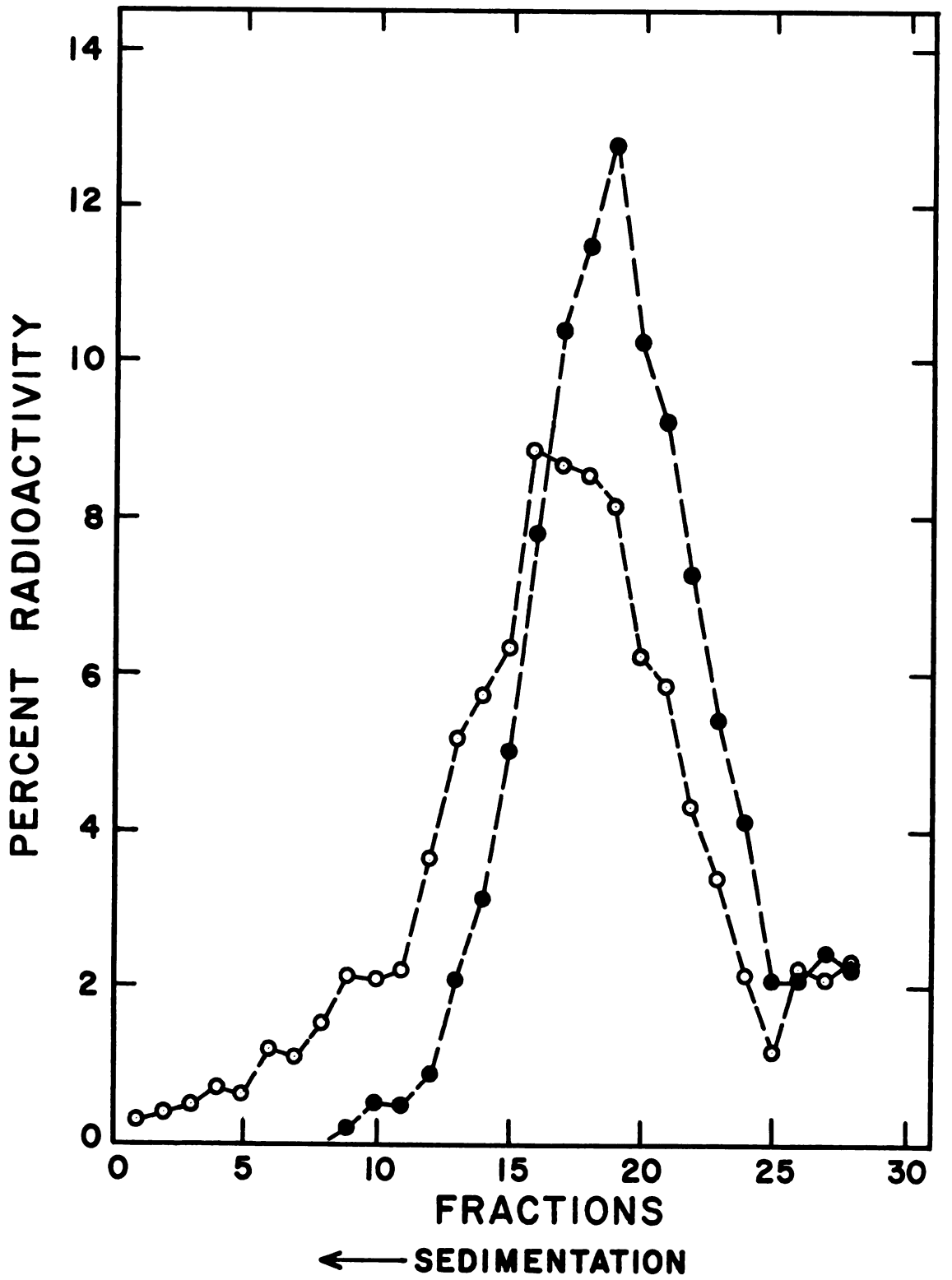
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Figure 36.

Figure 37. Sedimentation profiles of extracted DNA from human skin fibroblasts (736-NF) after treatment with M. luteus endonuclease. Confluent cells were incubated at 41°C for 72 hours in medium deficient in arginine and isoleucine before exposure to 20 J/m^2 ultraviolet radiation. Values of M_w were 5.55×10^6 (Fractions 7 to 25) for DNA extracted immediately after irradiation (solid circles) and 7.91×10^6 (Fractions 1 to 25) for DNA extracted 24 hours after irradiation (hollow circles)

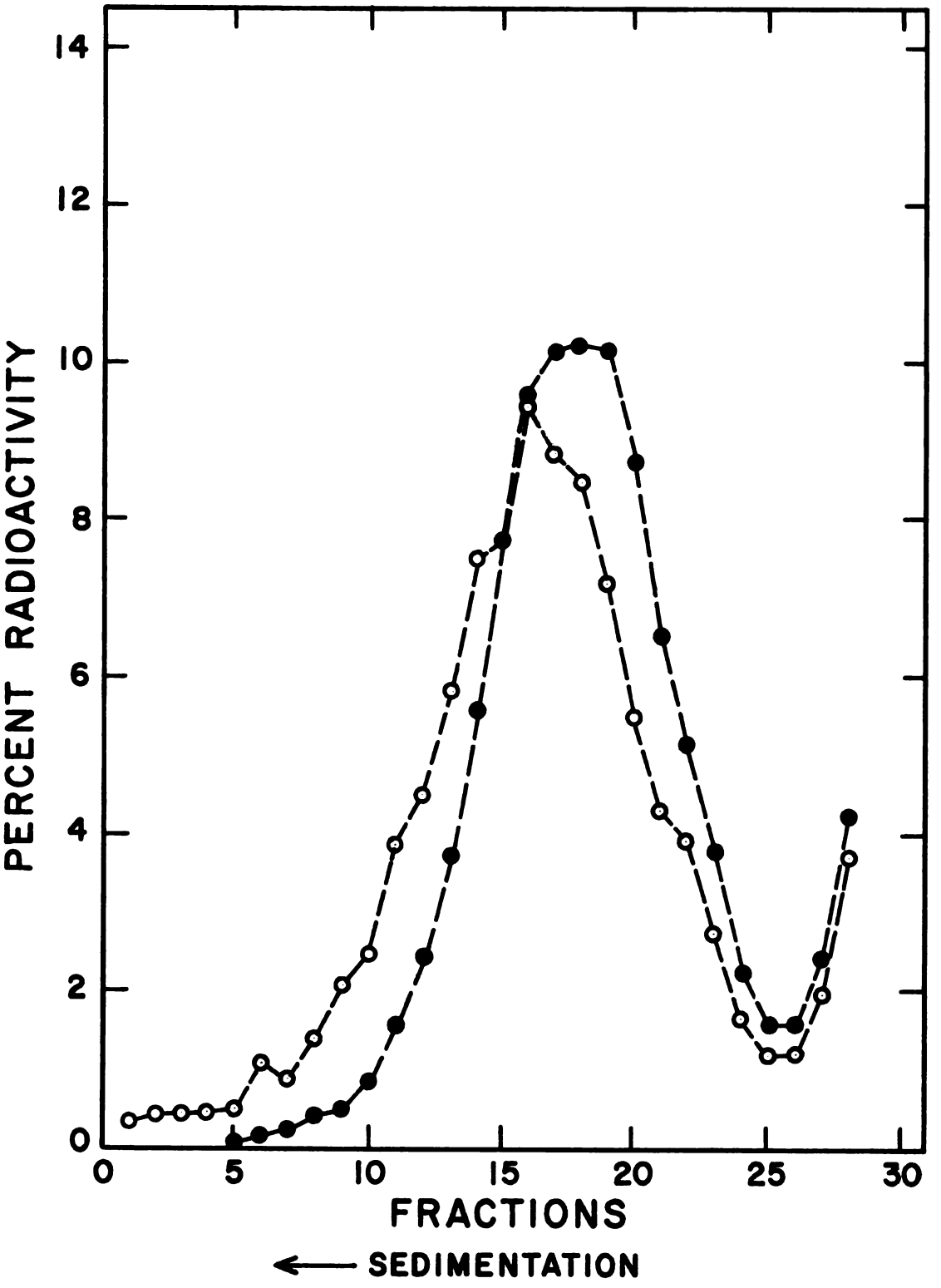
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Figure 37.

DISCUSSION

An exposure to 2.5 J/m^2 of UV radiation produced 5.4×10^{-8} endonuclease sensitive sites per dalton in V-79 cells. Other investigators observed 15.8×10^{-8} sites produced per dalton when their V-79 cells were exposed to 3.6 J/m^2 UV radiation[102].

A six hour post-UV repair period removed 2.1×10^{-8} sites per dalton or 39% of the sites produced by 2.5 J/m^2 UV. The other investigators observed 2.4×10^{-8} sites per dalton removed in a 9 hour period after UV irradiation at 2.5 J/m^2 [102]. Incubation of V-79 cells at 41°C for 3 days before 2.5 J/m^2 UV inhibited the removal of 0.74×10^{-8} sites per dalton or 35% of the sites.

A 24 hour period of post-UV repair resulted in the removal of 24.0×10^{-8} to 27.8×10^{-8} sites per dalton that were produced by 20 J/m^2 UV in Rid Mor cells that were not treated with hydroxyurea. Other investigators observed a removal of 28×10^{-8} sites per dalton in 24 hours when the same Rid Mor cells were exposed to 20 J/m^2 UV [103].

The results show that pre-UV hyperthermia at 41°C for 1 to 6 days inhibited the removal of endonuclease sites in the DNA of human fibroblasts. There is a tendency for this inhibition to increase with increasing duration of pre-UV hyperthermia for both Rid Mor and 736-NF human fibroblasts. Incubation at 41°C for 6 days before 20 J/m^2 UV either inhibited the 24 hour removal of endonuclease sites by 55% (in Rid Mor cells) or caused cell detachment (in 736-NF cells).

Hydroxyurea did not affect the amount of endonuclease sensitive sites produced by 20 J/m^2 UV radiation in Rid Mor cells. However, hydroxyurea, at a concentration of 5 m$\underline{M}$, inhibited the endonuclease site removal by approximately 50% in the same cells.

If the endonuclease sensitive sites are at the pyrimidine dimers produced by UV radiation, the pre-UV hyperthermia significantly inhibits the removal of the UV-induced dimers in both V-79 cells and human skin fibroblasts. Usually the excision repair of UV-induced dimers is thought to involve four general steps: incision, excision, polymerization, and ligation. The results of this experiment indicate that the pre-UV hyperthermia impairs the incision step more significantly than the other steps involved in excision repair. Since the DNA must be nicked at dimer sites before the dimer is removed in the excision step, a thermally damaged incision mechanism would result in less nicks at dimer sites, consequently inhibiting the removal of the dimers (endonuclease sites) in the excision step. This inhibition of endonuclease site removal is observed. The observation that cells incubated at either 37°C or 41°C before UV have no significant differences in the amount of breaks in their DNA, if allowed to incubate after UV, but not treated with M. luteus endonuclease, indicates that the incision step must be immediately followed by the other three steps. If one or more of the excision, polymerization, or ligation steps is thermally damaged at a faster rate than the incision step, then more breaks should have been observed in DNA from cells incubated at 41°C before UV. Therefore, the thermal damage of incision appears to be the rate limiting step in the thermal damage of the excision repair system. The incision mechanism in mammalian cells might involve a dimer specific endonuclease as in Micrococcus luteus. The thermal denaturation of this endonuclease might then be the rate limiting step in the thermal impairment of the excision removal of UV-induced pyrimidine dimers.

SUMMARY

Incubation of V-79 or human skin fibroblasts at 41°C for at least 3 days before UV irradiation significantly impaired the removal of sites sensitive to Micrococcus luteus endonuclease. Hydroxyurea had the same effect. The results indicate that thermal damage of the incision mechanism appears to be the rate limiting step in the thermal inhibition of excision repair.

CHAPTER V

DISCUSSION

THE CALCULATION OF THE ACTIVATION ENTHALPY FOR THERMAL IMPAIRMENT OF EXCISION REPAIR IN V-79 CELLS AND HUMAN SKIN FIBROBLASTS

When Chinese hamster cells or human skin fibroblasts were incubated at elevated temperatures for several days before UV irradiation, excision repair was significantly decreased. This impairment was observed whether repair was measured by unscheduled DNA synthesis, autoradiography, or by endonuclease site sensitivity. Since this effect takes place over a period of days, this process should have a relatively high activation enthalpy (compared with chemical reactions that take place within minutes). The results of the endonuclease site specificity experiments suggest that the rate limiting step for this effect might be the thermal denaturation of a dimer specific endonuclease. If the thermal impairment of excision repair involves the thermal denaturation of a protein, this process should obey the compensation law, given by Equation (3) of page 2 of this dissertation. If the denaturation of this protein follows first order kinetics, then this process should obey Equation (2), of page 1 of this dissertation, the absolute rate theory equation. Combining Equations (2) and (3) yields an equation that should be followed by a protein denaturation which obeys both the compensation law and first order kinetics:

Figure 38. Plots of Equations (24) from page 155 for temperatures (T) at 37°C, 40°C, and 41°C. The dotted lines represent the rate constant, $k_D(T)$, as a function of the activation enthalpy, $\Delta H^{\dagger}$. The solid curves represent the rate constant differences, $k_D(T) - k_D\ (37°C)$, for T at 40°C and at 41°C. The points represent data obtained from Table 3

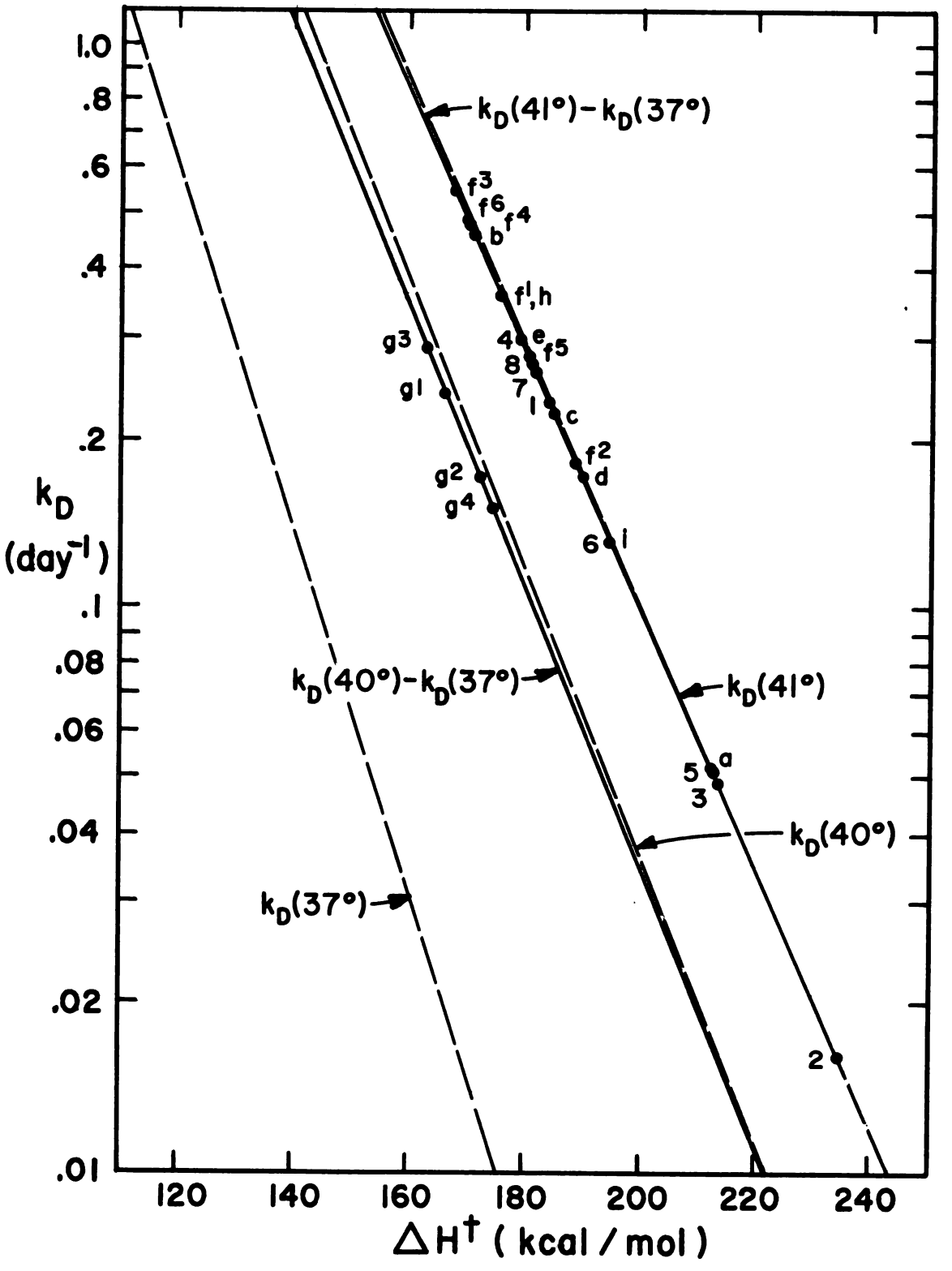
1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
.01
k_D (day^{-1})
120
140
160
180
200
220
240
$\Delta H^{\dagger}$ (kcal / mol)
$k_D(41°)-k_D(37°)$
$k_D(40°)-k_D(37°)$
$k_D(41°)$
$k_D(40°)$
$k_D(37°)$
f3
f6
b
f4
f1,h
g3
4
e
f5
8
7
1
c
g1
f2
d
g2
g4
6
i
5
a
3
2

Figure 38.

Table 3. The thermal inhibition of repair

Point on Figure 38	Figures in text	Incubation before ultraviolet irradiation: time t (days)	Incubation before ultraviolet irradiation: temperature T (°C)	Amount of repair (see text): N(T)	Amount of repair (see text): N(37°C)	Repair ratio $\frac{N(T)}{N(37°C)}$	Rate constant difference $k_\Delta(T)-k_\Delta(37°C)$ ($days^{-1}$)	Activation enthalpy $\Delta H^\dagger$ (kcal/mol)
a	2	0.875	41	43	45	.96	.052	213
b	3	0.875	41	50	75	.67	.46	171
c	4	3.0	41	79	154	.51	.22	186
d	5	3.0	41	50	83	.60	.17	191
e	6	3.0	41	10.2	24.8	.41	.30	180
f^1	8	0.5	41	90	108	.83	.36	176
f^2	8	1.0	41	90	108	.83	.18	189
f^3	8	1.5	41	47	108	.44	.55	168
f^4	8	2.0	41	41	108	.38	.48	171
f^5	8	2.5	41	54	108	.53	.28	181
f^6	8	3.0	41	25	108	.23	.49	170
g^1	16	3.0	40	41	85	.49	.24	166
g^2	16	3.0	40	163	272	.60	.17	172
g^3	16	3.0	40	125	295	.42	.29	162
g^4	16	3.0	40	225	342	.64	.15	174
h	22	3.0	41	5321	15548	.34	.36	176
i	25,26	3.0	41	1.4	2.1	.67	.13	196
1	18	3.0	41	700	1400	.50	.23	185
2	18	3.0	41	4100	4300	.95	.016	235
3	28,29	3.0	41	7.6	8.8	.86	.049	214
4	30,33	1.0	41	20.6	27.8	.74	.30	180
5	31,34	3.0	41	20.6	24.0	.86	.051	214
6	32,35	6.0	41	11.0	24.4	.45	.13	196
7	30,36	1.0	41	21.5	27.8	.74	.26	182
8	31,37	3.0	41	10.7	24.0	.86	.27	181

$$k_D = \frac{k_B T}{h} \exp\left[\frac{\Delta H^{\dagger}}{R}\left(\frac{1}{T_c} - \frac{1}{T}\right) + \frac{b}{R}\right] \qquad (24).$$

The Equations (24) are plotted in Figure 38 for temperatures (T) at 37°C, 40°C, and 41°C, with T_c = 325°K and b = -64.5 cal/mol °K, the constants for irreversible protein denaturation and single cell death. Since thermal inhibition of excision repair may involve irreversible protein denaturation and be related to cell death, this process of inhibition is expected to have the same constants.

When Equation (1) of page 1 of this dissertation is integrated, it yields

$$\ln (N_o/N) = k_D t \qquad (25).$$

If T_1 and T_2 are two different temperatures, the difference between the rate constants at these temperatures is

$$k_D(T_1) - k_D(T_2) = (1/t) \ln [N(T_2)/N(T_1)] \qquad (26).$$

The rate constant differences, $k_D(T) - k_D(37°C)$, are shown in Figure 38 for T = 40°C and T = 41°C. The data from all experiments showing an effect on repair with pre-UV hyperthermia are compiled in Table 3. The activation enthalpy, $\Delta H^{\dagger}$, is read from Figure 38 for corresponding rate constant differences.

The average activation enthalpy for V-79 cells is 179 kcal/mol, ranging from 162 to 213 kcal/mol. For V-79 cells, 16 out of 17 experiments lie within 179 ± 17 kcal/mol. For human skin fibroblasts, the average activation enthalpy is 199 kcal/mol, ranging from 180 to 235 kcal/mol.

EVIDENCE THAT THE DENATURATION OF THE DNA REPAIR PROTEINS IS A RATE LIMITING STEP IN THE THERMAL DEATH OF SINGLE CELLS

The results from unscheduled DNA synthesis and autoradiography indicate that a significant decrease in the amount of excision repair precedes death in V-79 cells, following an incubation period for three days at either 40°C or 41°C. This suggests that the thermal impairment of a DNA repair process, such as excision repair, is the rate limiting step for the thermal death of V-79 cells.

The results from endonuclease site sensitivity indicate that the thermal impairment of the incision step (perhaps the thermal denaturation of a dimer specific endonuclease) is the rate limiting step in the thermal damage of the excision repair process for both V-79 cells and normal human skin fibroblasts. Since the "repair ratios" and "thermal inhibition of repair" activation enthalpies (Table 3) were roughly the same whether repair was measured by ^{3}H-Thymidine incorporation or by endonuclease site specificity, it appears that the lower ^{3}H-Thymidine incorporation into DNA, observed after UV irradiation following 3 day incubation at 40°C or at 41°C, is primarily due to the thermal impairment of the incision step of excision repair, rather than due to the thermal impairment of the mechanisms for transport and phosphorylation of exogenous ^{3}H-Thymidine into the ^{3}H-dTTP which is to be incorporated into the damaged DNA.

Other investigators measured thermal death in cells by the loss of the ability of a cell to proliferate sufficiently to form a grossly visible clone in 1 week[16]. They determined the activation enthalpy for the thermal death in a subline of the V strain Chinese

hamster lung fibroblasts to be 185 kcal/mol. This value is very close to the activation enthalpies of "thermal inhibition of repair" for Chinese hamster lung fibroblasts (V-79) as compiled in Figure 38 and Table 3, suggesting that the thermal impairment of a DNA repair process, such as the incision step of excision repair, is a rate limiting step in the thermal death of Chinese hamster lung fibroblasts.

Although three day incubation of V-79 cells enhanced the rate of detachment (a characteristic of cell death) after this period, Figure 17 shows that ultraviolet irradiation at 15 J/m^2 did not affect the rate of detachment. This implies that a temperature sensitive mechanism other than excision repair must be necessary to keep the cells attached to the surface of the culture dish. The thermal damage to this mechanism must be the rate limiting step for cell detachment since the period of hyperthermia precedes cell detachment. However, the activation enthalpies for the thermal damage of this mechanism and excision repair must be roughly the same since the period of hyperthermia is the same for the effects of the thermal damage of these mechanisms to be expressed.

Other investigators measured the effect of hyperthermia on the repair of x-ray damage[104-106]. Although single- and double-strand breaks are not detectable after hyperthermia at temperatures between 41°C and 45.5°C[107], x-ray induced strand-break rejoining was found to be significantly inhibited by prior heating to these temperatures[104]. Furthermore, thymine base damage of the 5', 6'-dihydroxydihydrothymine (t') type is produced by hydroxyl radicals attacking the DNA during the x-irradiation of cells[108]. The

excision repair of the t'-type product from x-irradiated DNA has been shown in a number of cell lines[109]. Although it was shown that hyperthermia at 45°C did not produce an excess of t'-base damage in Chinese hamster ovary (CHO) cells and that 15 minute hyperthermia at 45°C before x-irradiation did not result in more base damage than in unheated cells, the excision of the t'-type products is inhibited by as much as 33% by preheating the CHO cells at 45°C for 15 minutes before x-irradiation[105]. The activation enthalpy was calculated by using Equations (24) and (28) and by assuming the compensation law. Using $N(45°C)/N(37°C) = 0.67$, $t = 15$ minutes, and $T = 45°C$, and assuming $k_D(37°C)$ as negligible, the activation enthalpy for the inhibition of the excision of the t'-type products in CHO cells becomes 184 kcal/mol. This value is very close to the activation enthalpy for thermal death in Chinese hamster lung fibroblasts and for the inhibition of UV-induced dimer excision repair in V-79 cells. Other investigators did not find an effect on repair when V-79 cells were heated to 42°C for two hours before x-irradiation[106]. However when Equation (24) was used with $T = 42°C$, $t = 2$ hours, and the activation enthalpy assumed to be 184 kcal/mol, $N(42°C)/N(37°C)$ became 0.95, an effect difficult to detect.

It was mentioned in Chapter I (p. 13) that there were at least five possibilities which could account for the decrease in repair with hyperthermia. Possibilities (2), (3), and (4) are shown to be inconsistent with the results from the experiments involving excision repair of UV-induced dimers. Regarding possibility (2), the manufacturing rate of repair proteins did not decrease after

hyperthermia since cycloheximide, an inhibitor of protein synthesis, did not inhibit unscheduled DNA synthesis in UV-irradiated or non-irradiated V-79 cells that were incubated for 3 days at either 37°C or 41°C. However, the manufacturing rate of the repair proteins may have decreased during the period of hyperthermia before cycloheximide was added. Regarding possibility (4), processes which damage the DNA do not have their rates increased more than the rate of repair since a three day incubation at 41°C does not produce more endonuclease sensitive sites in the DNA of cells that were UV-irradiated or non-irradiated. This result is also inconsistent with possibility (3); thus repair proteins are not converted into damaging proteins. However, both possibilities (1) and (5) are consistent with the results of the experiments. Not only might a dimer-specific endonuclease denature and repair ineffectively or not at all (1), but proteins in close proximity to the pyrimidine dimer may denature and interfere with the incision step in the excision repair process (5). It was reported that exposing cells to temperatures greater than 37°C causes a temperature- and time-dependent increase in the protein-to-DNA ratio of chromatin[109]. An excessive amount or protein adsorbed onto chromatin might render the DNA inaccessible to the function of the excision enzymes[105].

THE ACTIVATION ENTHALPIES OF THE DNA REPAIR PROTEINS DETERMINING THE MAXIMUM LIFE SPAN OF A MULTICELLULAR ORGANISM

The V strain of Chinese hamster cells was originally isolated from the lung tissue of Cricetulus griseus, the Chinese striped hamster[110]. The life spans of mice and rats, which are closely related to the hamster, follow the power law of Equations (5) and (6) up to

the age of 1000 days, with n = 5 [1]. Hamsters usually live up to two years and hibernate during the winter allowing their body temperatures to fall to 4°C. Also the species Cricetulus griseus is particularly susceptible to cancer and their cells tend to have greater amounts of chromosomal aberrations and polyploidy[111]. Thus Cricetulus griseus may spend only a year with a body temperature of 37°C.

If the power law is to be valid over this year, ρt should be less than one for t up to one year. This means that ρ should be less than 0.0027 day^{-1}. If ρ equals k_D, the rate constant for the rate limiting steps leading to the death of the hamster, then according to Figure 38, the activation enthalpy ($\Delta H^{\dagger}$) for the rate limiting steps should be greater than 193 kcal/mol. This value is high but within the range of activation enthalpies for thermal inhibition of excision repair as measured by unscheduled DNA synthesis in cells taken from Cricetulus griseus (V-79). Moreover, it is very close to the activation enthalpy of 196 kcal/mol for the thermal impairment of the incision step in excision repair as measured by endonuclease site specificity. Thus the thermal denaturation of a DNA repair protein, such as a dimer specific endonuclease, might be a rate limiting step in the death of Cricetulus griseus.

CHAPTER VI

CONCLUSIONS

As a living cell procedes through time, thermal fluctuations will tend to denature its proteins. Some of the proteins that are associated with DNA are potentially lethal to the cell when denatured, particularly those involved in repair. Thus a protein near a damaged portion of the DNA may denature and inhibit the repair of the damage, or a protein involved in the repair process may denature preventing any further repair of the damage from taking place. Unrepaired damage of the DNA might either be lethal to the cell or be carried along as a mutation to its daughter cells if the cell is allowed to divide. Hyperthermia enhances the rate of these protein denaturations, consequently promoting cell death.

One type of damage to DNA is exemplified by the production of pyrimidine dimers by ultraviolet radiation. In the excision repair of UV-induced dimers, the incision step is necessary before the other steps can follow. A protein denaturing near the dimer site might prevent the incision mechanism from producing a nick in the DNA near the dimer site; or a protein of the incision mechanism itself, such as a dimer specific endonuclease, might denature to a product unable to carry out incision at the dimer site. The dimer will persist until an intact incision mechanism is accessible to the dimer site. This might not occur until after the presence of the

dimer is expressed in a form that is lethal to the cell. For example, a dimer occuring at a segment of the DNA encoding for a protein might cause the segment to be mistranscribed. This might result in the production of a lethal protein or a useless protein in place of a protein that is necessary for cell survival. The dimer might also fall within a regulatory site interfering with cell proliferation. Since the effects of the presence of the dimer are immediate, the rate limiting step for these effects is the thermal denaturation of the protein that prevents the incision step in the excision repair of the dimer.

By stimulating protein denaturation with hyperthermia, it was shown that the thermal impairment of the incision step was the rate limiting step in the thermal inhibition of the excision repair of UV-induced dimers for both V-79 cells and human skin fibroblasts. By using the compensation law, it was shown that the activation enthalpy for thermal hindrance of the incision step was roughly equal to the activation enthalpy for the death of V-79 cells. Furthermore, thermal hindrance of excision repair was observed to precede death in the Chinese hamster fibroblasts. Thus it might be concluded that the thermal denaturation of a protein associated with the incision step of excision repair of ultraviolet radiation-induced pyrimidine dimers is the rate limiting step for one of the processes leading to the death of V-79 cells.

Unrepaired DNA in cells of multicellular organisms can perhaps lead to the death of the organism. This damage might transform a cell into a cancer cell; it might lead to the production of a substance that is lethal to the organism; or it might lead to the death of a few

cells that are necessary for the proper functioning of a control center within the organism. Thus the activation enthalpy for the denaturation of a protein associated with DNA repair might be an important factor for determining the life span of an organism. The evolution of a longer lived species might be accomplished by increasing the activation enthalpy for thermal denaturation of these DNA repair proteins and by increasing the replacement rate of these proteins.

Hypothesized DNA repair proteins, that may denature on the order of the life span of C. griseus, have an activation enthalpy close to the activation enthalpy for the thermal inhibition of the removal of endonuclease sensitive sites, when the compensation law is used to calculate these activation enthalpies. This suggests that the thermal denaturation of a DNA repair protein, such as a dimer specific endonuclease, is the rate limiting step for one of the processes leading to the death of the Chinese striped hamster.

CHAPTER VII

RECOMMENDATIONS

Further experiments can be conducted to relate the denaturation of DNA repair proteins and the thermal death of single cells. It was mentioned in Chapter V (p. 158) that two possibilities could account for the observed decrease in repair with pre-UV hyperthermia. One possibility is the thermal denaturation of a protein that is involved in the incision step of excision repair, such as a dimer specific endonuclease. Another possibility is the thermal denaturation of a protein in close proximity to the pyrimidine dimer site, which may interfere with the incision step of excision repair when denatured. Dimer-specific endonuclease from M. luteus can be heated at various temperatures before being assayed with UV irradiated DNA as a substrate. The first possibility is supported if the endonuclease pre-heated at higher temperatures made fewer breaks in the DNA during the assay.

If the cells were treated with a protein inhibitor, such as cycloheximide throughout the entire period of hyperthermia, an investigator could determine whether the manufacturing rate of the repair proteins decreased at the higher temperature. Furthermore, if cells were heated at three or more different temperatures above 37°C before ultraviolet irradiation, an activation enthalpy for the thermal inhibition of repair could be obtained directly, without the benefit of the

compensation law. The duration of the pre-UV hyperthermia should be long enough to observe an effect and short enough as to not cause the lethality of the cells. Ideally, cells inoculated from the same original culture should be heated simultaneously in three or more separate incubators set at different temperatures. This is because there appears to be some variation in the amount of excision repair after ultraviolet irradiation that may depend on the history of the culture from which the cells were inoculated, when the inoculation took place, or the concentration of the cells during inoculation.

More intensive studies of this above mentioned variation in excision repair should be conducted in order that this variation be controlled. Experiments can be conducted with other means of preventing "semi-conservative DNA replication" such as lowering the serum concentration of the medium. Since the amount of excision repair determined by the dimer-specific endonuclease assay is not masked by "semi-conservative DNA synthesis", experiments can be conducted with confluent cells that are not required to be maintained in a medium that is deficient in arginine and isoleucine. Since incubation in deficient medium might tend to reduce protein synthesis, experiments can be conducted with cells in media containing arginine and isoleucine to determine if DNA repair proteins are replaced. If they are replaced, pre-UV hyperthermia might affect the rate and amount of replacement.

The effect of pre-UV hyperthermia on excision repair can be observed in repair deficient mutants to determine if the repair proteins in the mutants are more sensitive to heat than those in normal cells. These experiments can be conducted with cells from other tissues and other organisms to determine if there is a variation in the thermal inhibition

of repair with different tissues and different organisms. A study can be conducted to determine if repeated UV irradiation can enhance the amount of excision repair. The effect of hyperthermia with repeated UV irradiation of DNA on excision repair can be determined. Finally, more extensive studies can be conducted on the effect of pre-treated hyperthermia on repair of damage caused by treatments other than UV irradiation, such as x-ray, gamma ray, or chemical treatment.

APPENDICES

Appendix A

The media used in the present investigation are designated as "C-15", "D", and "D" without arginine or isoleucine. Media "C-15" and "D" was prepared from a specially ordered 10 liter package of Eagle's minimal essential medium (EMEM) which had 50% increase of all the essential amino acids, except glutamine, 50% increase of all the vitamins, 100% increase of all the non-essential amino acids, but without glucose and phenol red (Gibco, Grand Island, NY). In addition, the 10 liter aqueous solution of "D" medium contained 1.1 g sodium pyruvate, 10 g glucose, 4.87 g sodium chloride, 10 g sodium bicarbonate. Medium "C-15" was prepared in the same manner as "D" except that 15 g sodium bicarbonate was used. In addition, "C-15" medium contained 10 mg phenol red powder, 50 mg hypoxanthine, 50 mg thymidine, and 50 mg uridine. Medium "D" without arginine or isoleucine was prepared in exactly the manner as "D" medium except that the specially ordered package of EMEM was deficient in arginine and isoleucine.

Appendix B

The average molecular weights of DNA from Rid Mor cells were calculated from a computer program from R. B. Setlow[72]. Table 4 contains a modified version of this program which was used to calculate the average molecular weights of DNA from 736-NF human skin fibroblasts. Only the range of fractions that appeared to lie within a random distribution of sizes of DNA was used in the calculation of the average molecular weight, M_w. The fractions used to calculate M_w are indicated in the captions of Figure 24 through Figure 37. Fractions that were included in both the high and low molecular weight tails of the distribution were usually not utilized in the calculations of M_w.

Table 4. A program to determine average molecular weights

```
      PROGRAM MWDETR (INPUT, OUTPUT, TAPE2=INPUT, TAPE6=OUTPUT)

  PROGRAM DETERMINES MOLECULAR WEIGHT DISTRIBUTIONS FOR A TRITIUM AND
CARBON-14 DOUBLE-LABELED SAMPLE.  MOLECULAR WEIGHT CALIBRATIONS ARE
FOR A BECKMAN SW56 ROTOR.  SPILLOVER COEFFICIENTS ARE USED TO
CORRECT OVERLAP IN THE TWO CHANNELS.  QUENCHING CORRECTIONS ARE NOT
MADE.

      INTEGER BKGRND
      DIMENSION WEIGHT (100), WWAVG(2), WNOAVG(2), PCT(2,100),
     1CHAN(2,100), BKGRND(2), PCTSUM(2), HEAD(7)

READ HEADER

      READ (2,99) HEAD

READ ROTOR RPM, CENTRIFUGATION TIME, LAST FRACTION (PORTION OF A
COMPLETE FRACTION), COUNT TIME (MIN.), AND SPILLOVER COEFFICIENTS
(CARBON CHANNEL FIRST)

      READ (2,100) RPM, RUNTIM, FRACT, TIM, SH, SC

READ BACKGROUND COUNTS

      READ (2,110) (BKGRND(I), I=1,2)

READ STARTING AND ENDING FRACTIONS TO BE ANALYZED

      READ (2,115) ISTRT, IEND
      KOUNT=0

READ COUNTS IN THE H-3 AND C-14 CHANNELS

      DO 20 J=1,200
      READ (2,120) ICH1, ICH2
      IF (EOF(2)) 30,10

CALCULATE COUNTS PER MINUTE

10    CPM1=(ICH1-BKGRND(1))/TIM
      CPM2=(ICH2-BKGRND(2))/TIM
```

Table 4 (continued)

```
CORRECT FOR OVERLAP

      CHAN(1,J)=CPM1-SC*CPM2
      CHAN(2,J)=CPM2-SH*CPM1
      IF (CHAN(1,J).LT.0.0) CHAN(1,J)=0.0
      IF (CHAN(2,J).LT.0.0) CHAN(2,J)=0.0
20    KOUNT=KOUNT+1
      STOP
30    CONST=(RPM**2)RUNTIM
      WRITE (6,600) HEAD,RPM,RUNTIM,FRACT,TIM,SH,ST,(BKGRND(I),I=1,2),
     +KOUNT,ISTRT,IEND

CALCULATE MOLECULAR WEIGHT DISTRIBUTION

      DO 40 I=ISTRT,IEND
      RAD=((KOUNT-I)+0.5+FRACT)/(KOUNT+FRACT)
40    WEIGHT(I)=(((2.3263E8/CONST)*ALOG((5.93+RAD*5.71)/5.93))**2.72)*
     +1.0E-6

CALCULATE MOLECULAR WEIGHT AVERAGES AND PERCENT DISTRIBUTIONS

      DO 50 I=1,2
      WWAVG(I)=0.0
      WNOAVG(I)=0.0
50    PCTSUM(I)=0.0
      DO 60 I=ISTRT,IEND
      DO 60 J=1,2
      WWAVG(J)=CHAN(J,I)+WEIGHT(I)+WWAVG(J)
      WNOAVG(J)=CHAN(J,I)/WEIGHT(I)+WNOAVG(J)
60    PCTSUM(J)=PCTSUM(J)+CHAN(J,I)
      DO 70 J=1,2

      WWAVG(J)=WWAVG(J)/PCTSUM(J)
70    WNOAVG(J)=PCTSUM(J)/WNOAVG(J)
      DO 80 I=1,2
      DO 80 J=ISTRT,IEND
80    PCT(I,J)=(CHAN(I,J)/PCTSUM(I))*100.

WRITE RESULTS

      DO 85 I=1,2
85    WRITE (6,610) I,WWAVG(I),WNOAVG(I)
      WRITE (6,620) HEAD
      DO 90 I=ISTRT,IEND
90    WRITE (6,630) WEIGHT(I),(PCT(J,I),J=1,2)
      STOP
```

Table 4 (continued)

FORMAT STATEMENTS

```
99    FORMAT (1X,7A10)
100   FORMAT (IX,F4.0,2X,F4.0,2X,F3.2,2X,F4.1,2(2X,F4.3))
110   FORMAT (1X,I4,2X,I4)
115   FORMAT (1X,I3,2X,I3)
120   FORMAT (6X,I5,1X,I5)
600   FORMAT (1H1/////10X,7A10////20X,*CENTRIFUGE SPEED-*,5X,F4.0,1X,
     +*THOUSAND RPM*//20X,*SPIN TIME -*,5X,F4.0,1X,*MINUTES*//20X,
     +*LAST FRACTION -*,5X,F3.2//20X,*COUNT TIME -*,5X,F4.1,1X,*MINUTES*
     +//20X,*SPILLOVER COEFFICIENTS*/25X,*TRITIUM -*,5X,F4.3/25X,
     +*CARBON -*,5X,F4.3//20X,*BACKGROUND*/25X,*CHANNEL 1 -*,5X,I4/
     +25X,*CHANNEL 2 -*,5X,I4//20X,*NUMBER OF FRACTIONS -*,5X,I3/25X,
     +*FIRST FRACTION ANALYZED -*,5X,I3/25X,*LAST FRACTION ANALYZED -*,
     +5X,I3/////)
610   FORMAT (//20X,*CHANNEL*,1X,I1//25X,+WEIGHT AVERAGE MOL. WT. -*,
     +G12.6,1X,*10E6*//25X,*NUMBER AVERAGE MOL. WT. -*,G12.6,1X,*10E6*
     +//)
620   FORMAT (1H1/////10X,7A10////20X,*MOL. WT.*,10X,*CHANNEL 1*,10X,
     +*CHANNEL 2*/20X,*X 10 E 6*,10X,*PERCENT*,12X,*PERCENT*//)
630   FORMAT (20X,G10.4,7X,G12.6,7X,G12.6/)
      END
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LIST OF REFERENCES

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