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Protein Synthesis in *Escherichia coli* During Recovery from Exposure to Low Levels of Cd^{2+}

R. S. MITRA

Environmental Cellular Chemistry Laboratory, Department of Environmental and Industrial Health, School of Public Health, The University of Michigan, Ann Arbor, Michigan 48109

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Exposure of *Escherichia coli* to 3 μM Cd^{2+} results in 84 to 95% of the cells losing their ability to form colonies on plates of nutrient agar. Transfer of the cells to Cd^{2+}-free liquid medium results in a recovery of colony-forming ability without significant synthesis of DNA. As an early event in recovery, the cells exhibit a rapid uptake of [^{3}H]leucine. Recovery and this incorporation are inhibited by chloramphenicol or rifampin. Sodium dodecyl sulfate-gel electrophoresis of proteins from recovering cells labeled with [^{3}H]leucine for 1 min indicated the synthesis of at least two classes of proteins with apparent molecular weights of 55,000 to 65,000. One class bound Cd^{2+} and was absent in untreated cultures. The other class of proteins, which did not bind Cd^{2+}, was synthesized at a rapid rate in recovering cells and may be a normal cellular protein.

The exposure of *Escherichia coli* to Cd^{2+} results in extensive single-strand breakage in DNA, and 84 to 95% of the cells lose their ability to form colonies on nutrient agar (11). Eventually the cells accommodate (11). They repair their damaged DNA, regain their ability to form colonies without a concomitant increase in cell number (10), and after a long lag, resume normal proliferative activities. Cadmium-exposed cells recover their colony-forming ability much faster when incubated in Cd^{2+}-free liquid medium (11). The recovery is insensitive to low concentrations of hydroxyurea (10), and the incorporation of [^{3}H]thymidine is insignificant during the recovery phase (11). Mutants which have a temperature-sensitive DNA ligase do not recover at the nonpermissive temperature (9); it appears that Cd^{2+}-exposed cells repair their DNA by ligation without insertion of nucleotides, and the increase in the number of colony-forming cells is not a result of cell division. The present paper reports that the recovery of Cd^{2+}-exposed cells is sensitive to chloramphenicol or rifampin, that proteins, including one fraction which binds Cd^{2+}, are synthesized during recovery, and that the Cd^{2+}-binding fraction exists transiently and is not present in untreated cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* B was cultured in a salts-glucose synthetic medium at 37°C (11). The method used for determining the number of CFU in the culture has been described previously (11).

Treatment of cells with Cd^{2+} and posttreatment conditions (recovery). Cells from a 15-h culture were harvested by centrifugation at room temperature, suspended in growth medium containing 3 μM Cd^{2+}, and incubated for 3 h at 37°C. To study the effect of postincubation in Cd^{2+}-free medium after exposure to Cd^{2+}, cells were harvested at room temperature, washed twice with fresh medium, and inoculated into fresh growth medium free of Cd^{2+}.

Incorporation of radioactive leucine during recovery of Cd^{2+}-exposed cells. The Cd^{2+}-exposed cells were inoculated in Cd^{2+}-free growth medium containing 0.065 μCi of [^{3}H]leucine per ml of medium. At different intervals during incubation, 3 ml of culture were mixed with 1 ml of cold 30% trichloroacetic acid. Samples were filtered through Whatman glass fiber filters and washed three times with cold 5% trichloroacetic acid, twice with alcohol, and once with an alcohol-ether mixture (3:1) (3). Acid-insoluble radioactivity was determined by counting the filters in a Packard Tri-Carb liquid scintillation spectrometer with toluene counting fluid (5.5 g of Permablend I per liter of toluene). To study the effect of inhibitors on protein synthesis, chloramphenicol (50 μg/ml) or rifampin (25 μg/ml) was included in the medium at the beginning of the recovery experiment. As a control in the inhibitor studies, an equivalent amount of solvent which was used to solubilize chloramphenicol (alcohol-water, 1:4) or rifampin (dimethyl sulfoxide-water, 1:4) was used (19).

Labeling of cells for a short time and analysis of protein by sodium dodecyl sulfate-gel electrophoresis. Three-ml portions of the culture removed at appropriate intervals during recovery of Cd^{2+}-exposed cells were labeled with 3 μCi of [^{3}H]leucine for 1 min at 37°C. Labeled cells were chilled and immediately mixed with 1 ml of cold 30% trichloroacetic acid. The resulting precipitates were collected by centrifugation and washed twice with cold 7% trichloroacetic acid and once with acetone at 0°C. In some cases, Cd^{2+}-exposed cells were allowed to recover for 30 min and then were labeled for 1 min with either [^{3}H]leucine or ^{109}Cd. In one set of experiments, ^{109}Cd was added to Cd^{2+}-exposed cells at zero time (before recovery), and the incubation was continued for 30 min. An untreated culture of growing cells was also treated with $^{109}CdCl_2$ for 1 min. For the identification of rapidly labeled metal-binding proteins, whole cells were used since treatment of a metalloprotein such as metallothionein with acid results in the dissociation of the metal (7). The method was essentially that used by West and Emmerson (21). Labeled cells were centrifuged immediately in the cold and were washed twice with ice-cold growth medium. The acid-insoluble product or the washed whole cell preparation was prepared for disc gel electrophoresis (22) by heating at 100°C for 5 min in a solution consisting of 0.0625 M Tris-hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10 mM dithiothreitol, and 0.001% bromophenol blue. Electrophoresis was carried out (10% separation gel and 3% stacking gel) by using the discontinuous buffer system of Laemmli (5); gels were sliced and radioactivity was determined after digestion with NCS

(Amersham) solubilizer. Molecular weights were estimated by the interpolation from plots of log molecular weight versus distance migrated with the following proteins as standards having the subunit molecular weights indicated: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000).

Analytical procedure. Protein was determined by the method of Lowry et al. (6), using bovine serum albumin as a standard.

Chemicals. Rifampin and chloramphenicol were obtained from Sigma Chemical Co. Crystalline $3CdSO_4 \cdot 8H_2O$ was the product of Allied Chemicals. Carrier-free $^{109}CdCl_2$ (at a concentration of 2 mCi/ml) and [4,5-^{3}H]leucine (58.5 Ci/mmol) were obtained from New England Nuclear Corp.

RESULTS

Incorporation of labeled amino acid during recovery from Cd^{2+} exposure. The rate of incorporation of labeled leucine into acid-insoluble product in cells recovering from exposure to Cd^{2+} is shown in Fig. 1B. Between 2 and 6 h of recovery, the rate appeared constant, although slower than that observed during the initial 2 h of recovery (Fig. 1B). A similar profile of incorporation was obtained with labeled cystine. Throughout the recovery experiment, the protein concentration remained constant at 38 ± 2 μg/ml of culture.

Effects of chloramphenicol on the recovery process. The Cd^{2+}-exposed cells failed to recover in the presence of 50 μg of chloramphenicol per ml. The incorporation of labeled leucine was inhibited (Fig. 1B), and the cells gradually lost further colony-forming ability (Fig. 1A). Under the same experimental conditions, untreated cells retained colony-forming ability after 6 h of incubation, although the incorporation of leucine was inhibited. The inhibitory effects of chloramphenicol suggest a requirement for protein synthesis during recovery.

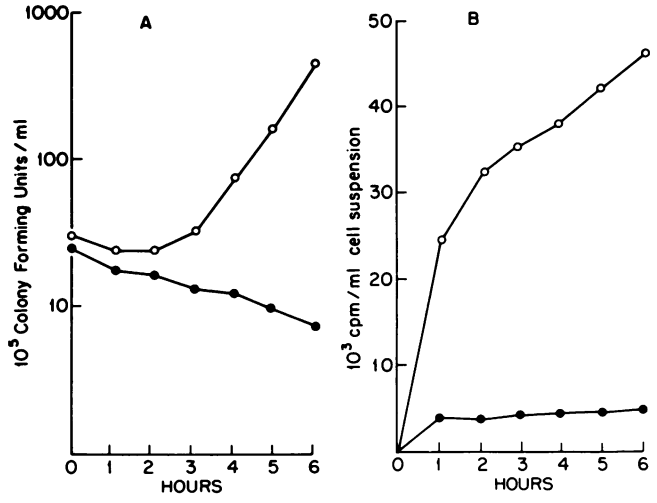

FIG. 1. Effect of chloramphenicol on recovery of *E. coli* from the effect of Cd^{2+}. Growing cells (4.1×10^7 per ml) were inoculated into 3 μM Cd^{2+}-containing medium and incubated at 37°C for 3 h. After 3 h of exposure, the number of CFU per milliliter of culture was 4.5×10^6. The bacteria were then harvested, washed twice with fresh medium, suspended in medium containing [^{3}H]leucine (0.065 μCi/ml), and incubated in the presence of 50 μg of chloramphenicol per ml (●) or in its absence (○). CFU (A) and radioactivity in acid-precipitable material (B) were determined.

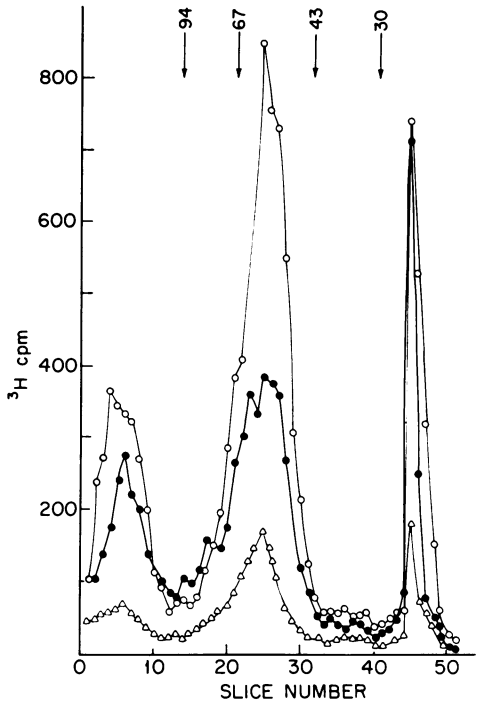

FIG. 2. Effect of rifampin on the synthesis of proteins during recovery of Cd^{2+}-exposed cells. Bacteria (3.1×10^8/ml) were treated with 3 μM Cd^{2+}, and after a 3-h exposure, the number of CFU per milliliter of culture was 2×10^7. The treated cells were then freed from Cd^{2+} by washing, suspended in fresh medium, and divided into two portions. One portion of the culture (6×10^6/ml) was labeled at 37°C for 1 min with [^{3}H]leucine after 30 min of recovery at 37°C (○), and the other half (7×10^6/ml) was incubated in the presence of rifampin (25 μg/ml) and labeled as before after an incubation of 30 min (●) or 1 h (△). The labeling was stopped by trichloroacetic acid, and the whole protein mixtures were analyzed by SDS-gel electrophoresis. Numbers at the top are molecular weights ($\times 10^3$) of the standards.

SDS-polyacrylamide gel electrophoresis of labeled leucine-incorporated proteins. Disc gel electrophoretic analysis of leucine-labeled proteins synthesized by Cd^{2+}-exposed cells during recovery (refer to Fig. 1B, 1-h incubation) indicated the presence of low-molecular-weight labeled proteins. However, gel electrophoresis of proteins from a culture of Cd^{2+}-exposed cells labeled for 1 min with [^{3}H]leucine at 30 min of recovery (i.e., before there was any increase in colony-forming ability) indicated the presence of two newly formed components (Fig. 2). One protein fraction having a molecular weight of >100,000 (gel slice number 2 to 10), contained ca. 10 to 15% of the initial incorporated radioactivity. The other fraction, obviously consisting of more than one component, appeared in the molecular weight range of 55,000 to 65,000 (gel slice number 18 to 30) and constituted ca. 45 to 55% of the labeled proteins. Proteins with a molecular weight ranging from 28,000 to 50,000 (gel slice number 30 to 40) were nearly absent when compared with the radioactive profile obtained in the untreated cells labeled with [^{3}H]leucine for 1 min under the same experimental conditions (Fig. 3). The radioactivity in high-molecular-weight components (>100,000 and 50,000 to 65,000) which appeared as a result of labeling with [^{3}H]leucine for 1 min in recovering cells appeared in proteins of low molecular weight within 30 min of further incubation after addition of

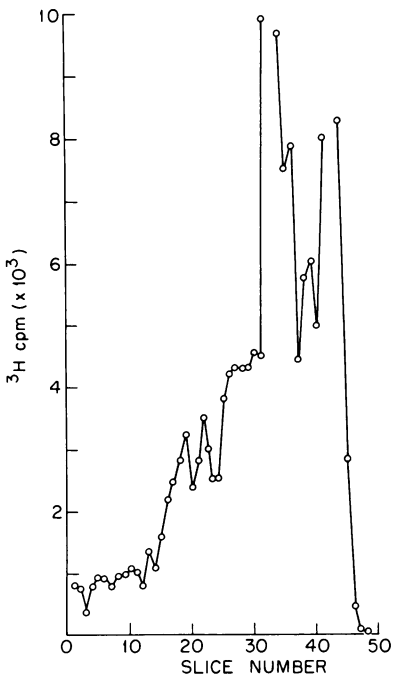

FIG. 3. Labeling of untreated cells for a short time and analysis of proteins by SDS-gel electrophoresis. A growing culture (8×10^7/ml) was labeled for 1 min with [^{3}H]leucine at 37°C and treated with trichloroacetic acid, and the whole protein mixtures were analyzed by SDS-gel electrophoresis.

excess nonradioactive leucine (1 mM). Similar conversion was observed when the radioactive precursor was removed quickly by centrifugation after a 1-min exposure to [^{3}H]leucine and the cells were further incubated for an additional 30 min.

Synthesis of rapidly labeled proteins in the presence of chloramphenicol or rifampin. Chloramphenicol prevented the recovery of the Cd^{2+}-exposed cells. Cd^{2+}-exposed cells were inoculated into fresh growth medium containing 50 μg of chloramphenicol per ml, and at different time intervals, a portion of the culture was exposed to labeled leucine for 1 min and the radioactivity in acid-insoluble product was analyzed by SDS-gel electrophoresis (data not shown). The overall level of radioactive incorporation was reduced by 85% after only 30 min of exposure to chloramphenicol. The synthesis of the low-molecular-weight components was decreased by ca. 55 to 60%, and inhibition increased with longer exposure to the drug. Similar results were obtained with 25 μg of rifampin per ml (Fig. 2). The synthesis of the lower-molecular-weight materials seemed to be less drastically affected by rifampin than by chloramphenicol. The Cd^{2+}-exposed cells failed to recover in the presence of 25 μg of rifampin per ml, and the cells gradually lost their ability to form colonies (data not shown).

Association of ^{109}Cd with the newly synthesized proteins during recovery. Since it has been reported (11) that a considerable amount of Cd^{2+} was found to be associated with the unaccommodated cells, it is possible that the rapidly synthesized proteins are capable of binding the Cd^{2+} so that the cells can compartmentalize the toxic ion and continue normal functions. To test the possibility that the newly synthesized proteins bind Cd^{2+}, Cd^{2+}-exposed cells were allowed to recover for 30 min and then were labeled for 1 min with ^{109}Cd; alternatively, ^{109}Cd was added to Cd^{2+}-exposed cells at zero time (before recovery), and the incubation was continued for 30 min. The results of this experiment are shown in Fig. 4. A striking difference between the radioactivity profile of acid-insoluble material and that of the whole cell is the absence of the high-molecular-weight component (>100,000) in the latter case. From the radioactive distribution of ^{109}Cd and ^{3}H, it is evident that the higher percentage of the rapidly labeled protein (Fig. 4a, peak A) is not associated with ^{109}Cd, whereas most of the labeled cadmium (Fig. 4b, peak C) is found to be associated with a rapidly synthesized protein (Fig. 4a, peak B) which always appears as a shoulder. Another cadmium-containing (Fig. 4b, peak D) rapidly synthesized protein is also present. Its synthesis largely depends on the length of incubation during recovery of Cd^{2+}-exposed cells, and it becomes the major Cd^{2+}-containing protein after 3 h of incubation. No ^{109}Cd was found to be associated with peaks C and D (Fig. 4b) when untreated cells were processed under identical experimental conditions. Although Cd^{2+} is associated with untreated cells, the binding appears to be nonspecific in nature and sticks at the top of the gel.

DISCUSSION

During recovery of Cd^{2+}-exposed cells, the synthesis of protein was rapid and did not show any lag, but followed complex kinetics. When there was a rapid synthesis of protein during the first 2 h of incubation, the Cd^{2+}-exposed cells were still losing their colony-forming ability. After that period, when the rate of protein synthesis had decreased, the cells were regaining their ability to form colonies. The early time seems to represent a "tooling up" period for recovery. That recovery of colony-forming ability was sensitive to chloramphenicol suggests that newly synthesized proteins are involved in the recovery process (12). The recovery of Cd^{2+}-exposed cells was also sensitive to the antibiotic rifampin, which is a specific inhibitor of the DNA-dependent RNA polymerase in bacteria and is known to affect synthesis of most species of RNA by inhibiting initiation of RNA chains (14). The failure of Cd^{2+}-exposed cells to recover in the presence of chloramphenicol or rifampin suggests that the synthesis of proteins is necessary for recovery. The SDS-gel electrophoretic analysis of acid-insoluble materials obtained after labeling with [^{3}H]leucine for 1 min indicates the transient presence of two classes of protein with molecular weights of >100,000 and 55,000 to 65,000. These two rapidly labeled components (>100,000 and 55,000 to 65,000) are most prominent in the first hour of incubation. They are formed in the presence of Cd^{2+}, although at a much slower rate. The synthesis of other cellular proteins with approximate molecular weights of 28,000 to 50,000 found normally was completely inhibited. It is not known at present whether the 55,000 to 65,000 proteins are present in normal cells, since it is difficult to identify them in the presence of other labeled proteins.

Unaccommodated cells contained a considerable amount of Cd^{2+}, and to recover from Cd^{2+} injury and resume normal metabolism, it has been proposed that the Cd^{2+} must be compartmentalized in the cell (11). This type of reasoning led us to investigate the Cd^{2+}-binding capacity of rapidly synthesized protein. Analyzing for ^{109}Cd by SDS-gel electrophoresis with whole cell homogenates (Fig. 4b) revealed the presence of two Cd^{2+}-containing proteins (peaks C and D). The relationship between these two proteins has not yet been established. The failure of control cells to bind ^{109}Cd under identical experimental conditions ruled out the possi-

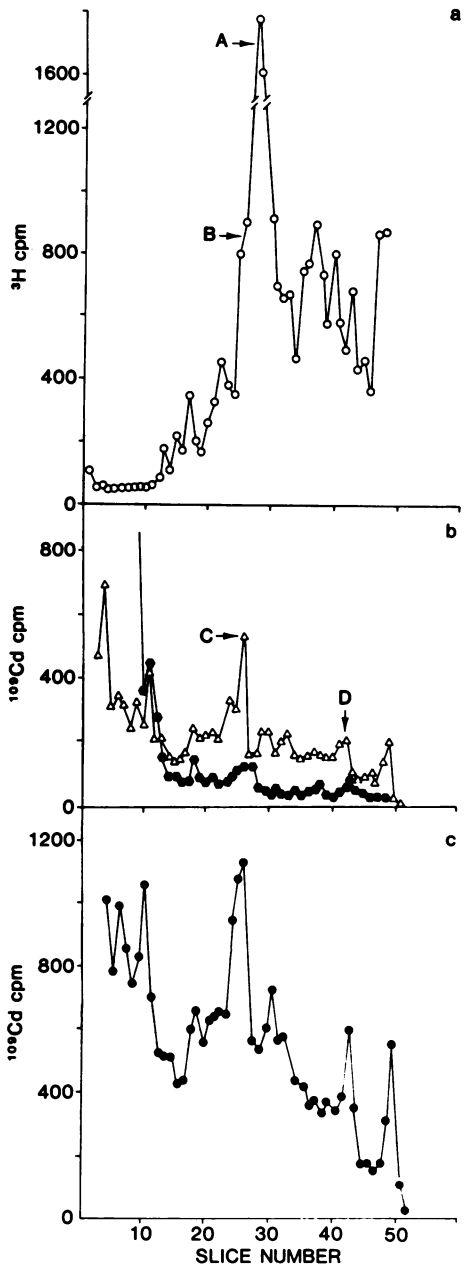

FIG. 4. Association of labeled cadmium with the newly synthesized proteins during recovery of *E. coli* from Cd^{2+} exposure. A culture (2.6×10^8/ml) was treated with 3 μM Cd^{2+}. After a 3-h exposure, the number of CFU per milliliter of culture was 4×10^7. The treated cells were then freed from Cd^{2+} by washing and were suspended in fresh medium. After a 30-min incubation at 37°C, 3 ml of culture was labeled for 1 min with (a) [^{3}H]leucine (○) or (b) ^{109}Cd (3.5×10^5 cpm) (△); since carrier-free ^{109}Cd was used, the concentration of cadmium was insignificant as compared to the 3 μM challenging dose. A 3-ml portion of a rapidly growing untreated culture (1.5×10^8/ml) was labeled with (b) ^{109}Cd (3.5×10^5 cpm) for 1 min at 37°C (●). In (c), ^{109}Cd (3.5×10^5 cpm) was added at zero time to 3 ml of culture, and the incubation was continued for 30 min (●). After being labeled with either ^{3}H or ^{109}Cd, the washed cells were dissolved in a small volume of lysing buffer and analyzed by SDS-gel electrophoresis.

bility that this binding was nonspecific in nature. Moreover, similar methodology was used by Riordan and Gower (13) for the identification of low-molecular-weight copper proteins in "copper-loaded liver", Rozalski and Wierzbicki (15) for the determination of the extent of mercury binding by chromatin, and Yamazaki (23) for the identification of selenium moiety in hydrogenase from *Methanococcus vannielii*. It is not possible to elucidate the nature of binding between the metal and the protein unless complete amino acid analysis of the induced purified protein is available. Whatever might be the nature of the bond, it is extremely stable, since the acid-insoluble materials were boiled in SDS-containing solution before electrophoresis, which destroys the tertiary structure of proteins.

The cadmium-binding species synthesized in response to Cd^{2+} exposure are significantly larger than metallothionein (4), which is synthesized de novo by eucaryotes after intracellular accumulation of the metal. Metallothionein is known to bind and to be induced by other metals such as mercury, zinc, and copper (4); the appearance of these proteins during recovery from Cd^{2+} exposure does not represent a general response to heavy metals. When cells were exposed to 3 μM Hg^{2+}, no growth was observed after 5 days of incubation. However, after 3 h of exposure (the number of colony-forming cells was 0.01%), the Hg^{2+}-exposed cells recovered after 10 h of incubation in fresh growth medium. At different times during recovery, 3 ml of culture was labeled with [^{3}H]leucine for 1 min; the acid-insoluble material was processed, and upon gel electrophoresis, the radioactive profile was no different from the profile obtained with untreated cells. The appearance of radiolabeled proteins was associated with the change in the number of colony-forming cells in contrast to Cd^{2+}-exposed cells, in which the protein synthesis was always followed by the change in the number of colony-forming cells. The above observations imply that the rapidly synthesizing proteins may be related to the recovery from Cd^{2+} exposure. It seems that the mechanism of accommodation to Hg^{2+} may be different from that to Cd^{2+}, although Hg^{2+} ions are known to bind many sulfhydryl-containing proteins in the cell (18) as well as to DNA and its components (2). It has been reported that accommodation to mercury and organic mercurials in microorganisms is a result of enzymatic detoxification and volatilization (17, 20).

The appearance of rapidly labeled proteins (Fig. 4a, peak A) which do not bind with cadmium may be a prerequisite for recovery of the Cd^{2+}-exposed cells. At present, neither the specific role nor the intracellular localization of these proteins is known. The synthesis of these two classes of proteins continues in the presence of hydroxyurea during recovery and suggests that they are not involved in the initiation of cell division. Induced synthesis of proteins appears to occur specifically under conditions in which the cellular DNA has been damaged (10), and they might be involved in protecting the single-stranded DNA from further nuclease activity. In *E. coli* one major protein, the *recA* gene product X, is synthesized in great abundance in response to agents which damage DNA or inhibit its synthesis (1). Protein X (M_r = 40,000), found mostly in the cytoplasm, was said to protect single-stranded DNA from degradation by *recBC* nuclease (16).

It appears that the induced synthesis of specific proteins in cells in response to different kinds of stress is an inherent property of the cell. For example, four proteins with molecular weights of 26,000, 29,000, 34,000, and 36,000 were synthesized in response to DNA damage by UV irradiation

in *Micrococcus radiodurans*, and it was proposed that these proteins participate in DNA repair (1). Other proteins whose synthesis is transiently induced by shift-up of growth temperature in *Drosophila*, mammalian cell cultures, bacteria, and yeasts have been demonstrated (8). The synthesis of heat shock proteins may fulfill some essential cellular function to protect the cell from thermal injury.

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