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# Four Proteins Synthesized in Response to Deoxyribonucleic Acid Damage in *Micrococcus radiodurans*

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Four proteins, α, β, γ, and δ, preferentially synthesized in ultraviolet light-treated cells of *Micrococcus radiodurans*, were characterized in terms of their molecular weights and isoelectric points. Within the sublethal-dose range, the differential rate of synthesis for these proteins increased linearly with the inducing UV dose. The degree of induction reached 100-fold, and the most abundant protein, β, amounted to approximately 2% of the total newly synthesized protein after irradiation. Damage caused by ionizing radiation or by treatment with mitomycin C also provoked the synthesis of the four proteins. The proportions between the individual proteins, however, varied strikingly with the damaging agent. In contrast to treatments which introduced damage in the cellular deoxyribonucleic acid, the mere arrest of deoxyribonucleic acid replication, caused by nalidixic acid or by starvation for thymine, failed to elicit the synthesis of either protein. Repair of deoxyribonucleic acid damage requires that a number of versatile and efficient processes be employed. It is proposed that the induced proteins participate in deoxyribonucleic acid repair in *M. radiodurans*. Mechanisms are discussed which would allow a differentiated cellular response to damages of sufficiently distinctive nature.

Protein synthesis in the period subsequent to irradiation is essential for maximal survival of both bacterial and mammalian cells (2, 5, 6, 31). In the most thoroughly studied system, *Escherichia coli*, one major protein, the *recA* gene product protein X (8, 10, 17, 22, 23), is synthesized in great abundance in response to agents which damage DNA or inhibit its synthesis. Mutant strains (*recA*, *lexA*) unable to derepress the synthesis of this protein are highly sensitive to the lethal action of radiation, and furthermore, cannot be mutagenized by UV light (16, 41). The precise function(s) of the induced protein is not known. Evidence has earlier been obtained for an indirect action by way of proteolytic cleavage of repressors (32, 41), whereas recent work has demonstrated and emphasized the direct participation of the protein in the process of genetic recombination (15, 24, 35).

The exceptional resistance of *Micrococcus radiodurans* to both UV and ionizing radiation has prompted much experimentation with this organism. Evidence has accumulated for a variety of efficient and accurate DNA repair mechanisms in *M. radiodurans* (1, 3, 12, 25, 26). No mutagenic effect of UV light was found in *M. radiodurans* (38) in contrast to the UV-induced, protein X-mediated, error-prone repair of wild-type *E. coli*. This paper examines the pattern of protein synthesis subsequent to irradiation of *M. radiodurans* to assess the importance of specifically induced, de novo-synthesized proteins for the error-free repair characteristic of this species.

## MATERIALS AND METHODS

**Growth, irradiation, and radiolabeling.** Wild-type *M. radiodurans* was grown at 37°C in Casamino Acids-supplemented medium, and growth was followed turbidimetrically (12). Thymine (5 μg/ml) was included for growth of the thymine-requiring strain MH 133 (12). All experiments were performed with exponentially growing cultures which had reached densities of $2.0 \times 10^7$ to $3.0 \times 10^7$ colony-forming units per ml. To remove unlabeled methionine, 10-ml samples were spun down, washed, and suspended in cold minimal medium supplemented with a synthetic amino acid mixture (20 μg/ml each) without methionine, tryptophan, or tyrosine. The suspended culture was transferred to a petri dish and irradiated with UV light (254 nm). The UV flux was determined by means of a radiometer (model IL 570; International Light). For experiments with ionizing radiation, the resuspension in a screw-cap tube was immediately passed onto the conveyer of a 10-MeV electron accelerator. The dose administered was measured calorimetrically. The number of viable cells was determined by plating on tryptone-glucose-yeast extract agar plates (12) before and after irradiation. Immediately after irradiation L-[$^{35}$S]methionine (30 to 80 μCi; 700 to 1,200 Ci/mol; Amersham Radiochemical Centre) was added, and the culture was shaken at 37°C for 30 min. Labeling was terminated by a chase of nonradioactive methionine (50 μg/ml) for 5 min. Cells were spun down, and the pellet was stored overnight at −20°C.

**Gel electrophoresis.** The frozen pellet was suspended in 100 μl of 10 mM Tris-hydrochloride–5 mM $MgCl_2$, pH 7.4, containing 50 μg of pancreatic RNase per ml. Cells were disrupted by sonication in three 30-s bursts (Ultrasonics Rapidis 180, microtip). Pancreatic DNase was added to 50 μg/ml, and the sample was left on ice for at least 15 min, urea and detergent were added (28), and the tube was centrifuged (15,000 × *g* for 10 min). Less than 10% of the radioactivity in the sonic extract sedimented with the pellet. This was taken to indicate a near complete cell breakage. The supernatant was subjected to two-dimensional electrofocusing and SDS-polyacrylamide gel electrophoresis essentially by the method of O'Farrell (28), except that the nonionic detergent Dawfax N9N (K. E. Dan ApS, Copenhagen) was used for the electrofocusing dimension in place of Nonidet P-40. The second-dimension separation was performed in uniform 12.5% polyacrylamide gels. After electrophoresis, gels were immersed in 3% glycerol for 15 min and then dried under vacuum. The position of labeled proteins was determined by radioautography (Kodak X-Omat MA), and the relevant spots were cut out and solubilized by hydrogen-peroxide and NCS solubilizer (Amersham) by the method of Pedersen et al. (30). The efficiency of scintillation counting of labeled proteins embedded in the gel and subsequently solubilized was reduced by 45% when compared with counting a sample in scintillation liquid containing Triton X-100 (13). All results have been corrected accordingly. Marker proteins, when present, were localized by staining with Coomassie brilliant blue (11).

## RESULTS

**UV light-induced proteins.** The overall rate of protein synthesis during a 30-min period after irradiation of *M. radiodurans* was little affected by UV doses in the range which left more than 10% of the cells viable. The distribution between individual proteins was, however, markedly changed. The majority of proteins was formed in diminished quantities after UV irradiation, whereas four proteins, present in inconspicuous amounts in unirradiated cells, became prominent (Fig. 1A-C). These proteins, somewhat arbitrarily and without their function being

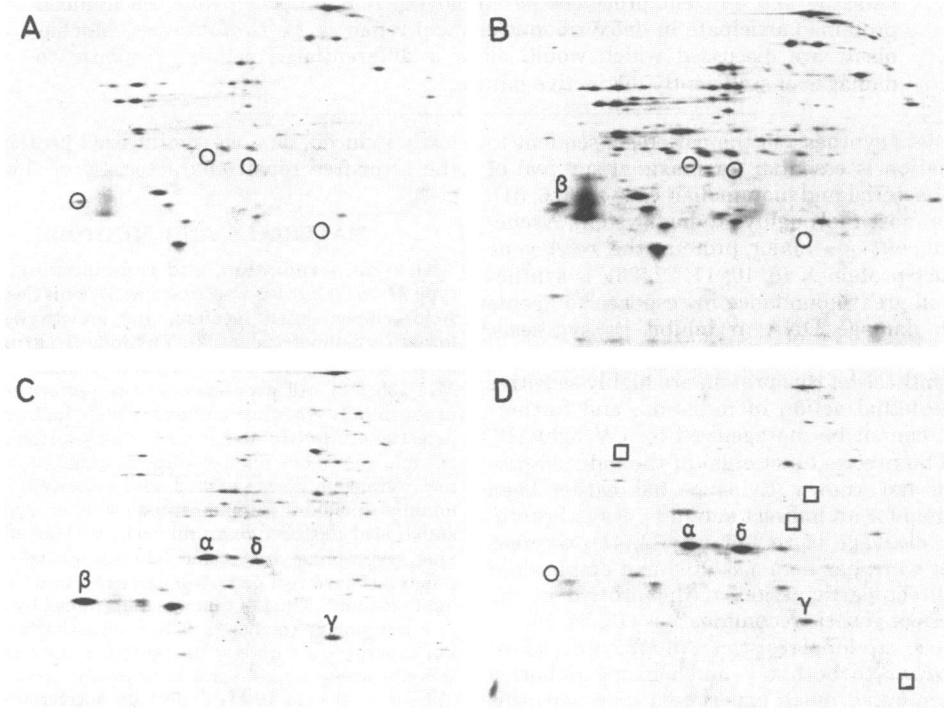


FIG. 1. *Autoradiograms from lysates of M. radiodurans subjected to two-dimensional electrophoresis. In the first dimension, proteins are displayed in order of increasing acidity from left to right. In the second dimension, proteins have been separated by size (28). Induced proteins α, β, γ, and δ have been indicated, or where present in amounts undetected by the film, their characteristic positions have been circled. In D, the positions of stained marker proteins (□) have been indicated; in order of decreasing size: bovine serum albumin, ovalbumin (hen), RNA polymerase subunit α (E. coli), and trypsin inhibitor (soybean). (A) Untreated control culture: load 1.8 × $10^5$ cpm, exposure time 64 h. (B) Control culture: load 1.8 × $10^5$ cpm, exposure time 309 h. (C) Culture exposed for 2 min to UV light at 2.0 W/$m^2$; 45% of the cells survived this dose. Load 3.3 × $10^5$ cpm, exposure time 64 h. (D) Culture exposed to 0.3 megarads of ionizing electron radiation. This dose caused no measurable cell death. Load 3.8 × $10^5$ cpm; exposure time 140 h.*

known, have been designated $\alpha$, $\beta$, $\gamma$, and $\delta$. Their molecular weights and isoelectric points (Table 1) were determined on the basis of marker proteins added to cell lysates before two-dimensional electrophoresis (e.g., Fig. 1D).

The relative rate of synthesis for proteins $\alpha$, $\beta$, $\gamma$, and $\delta$, i.e., the fraction of total synthesis devoted to these specific proteins, is a function of the UV dose given to the cells (Fig. 2). Protein $\beta$ is the only one of the set synthesized in readily measurable quantities in unirradiated cells (Fig. 1A and B). The basal level for $\beta$, 0.067% of total protein synthesis, increased to 1.9% after a UV dose leaving 45% of surviving cells, i.e., a relative induction of some 30-fold. Protein $\alpha$ was present in such minute quantities in unirradiated cells that heavy overexposure was needed to make visible a spot at the position of $\alpha$ (Fig. 1A and B). The protein thus disclosed may represent the basal level of $\alpha$ or, alternatively, could be a minor unrelated protein species. Such a protein would be completely obscured by $\alpha$ in irradiated cells. The basal level of 0.014% for $\alpha$ may therefore be a high value leading to a minimum estimate for the relative induction of 95-fold in irradiated cells. The basal levels of $\gamma$ and $\delta$ could not be spotted on the X-ray film, nor could any radioactivity above background be detected in the scintillation counter. UV irradiation resulted in synthesis of these two proteins to reach some 30-fold above the level of detection.

The specific activity of methionine, and thus the absolute rate of protein synthesis, during the period of incorporation is not precisely known because of incomplete removal of nonradioactive methionine present in the growth medium before irradiation (see Materials and Methods). However, the fraction incorporated after 30 min was, in all experiments, between 15 and 30% of the total amount of label added. There was no trend to indicate a difference between control cells and cells irradiated to leave upwards of 30% viable colony-forming units. Thus the doses of

TABLE 1. *Molecular weights and isoelectric points of induced proteins*[a]

| Protein | Mol wt | pI |
|---|---|---|
| $\alpha$ | 36,000 | 5.7 |
| $\beta$ | 29,000 | 6.4 |
| $\gamma$ | 26,000 | 5.0 |
| $\delta$ | 34,000 | 5.4 |

[a] Molecular weights were determined by the method of Weber and Osborn (39), whereas pI's were taken from the linear interpolation between soybean trypsin inhibitor (pI = 4.5), hen ovalbumin (pI = 4.8; 4.9), and bovine serum albumin (pI = 5.9; 6.0). The isoelectric points used for bovine serum albumin pertain to gels containing 6 M urea (33).

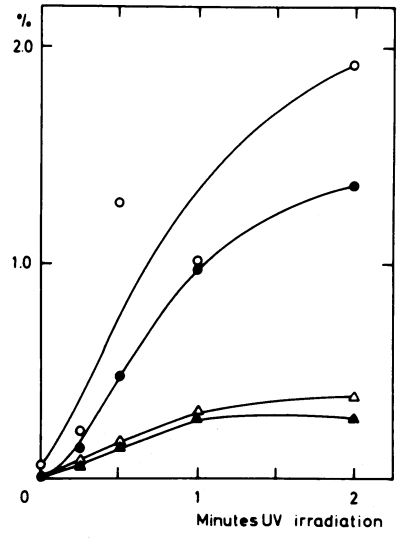


FIG. 2. *Differential rate of synthesis of proteins induced by UV light. The rates of synthesis after UV irradiation at 2.0 W/m$^2$ are expressed in percentage of total [$^{35}$S]methionine incorporated during the labeling period. Symbols: ●, $\alpha$; ○, $\beta$; ▲, $\gamma$; △, $\delta$.*

radiation employed did not diminish or only slightly diminished the rate of total protein synthesis during the first 30 min after irradiation. Consequently the relative inductions actually measured for proteins $\alpha$, $\beta$, $\gamma$, and $\delta$ (Fig. 2) represent absolute inductions of similar magnitude.

**Effect of ionizing radiation, mitomycin C, and arrest of DNA replication.** Ionizing electron radiation induced the same four proteins $\alpha$, $\beta$, $\gamma$, and $\delta$ (Fig. 1D). Proteins $\alpha$ and $\gamma$ were synthesized in similar amounts in cells irradiated with electrons and in cells treated with UV light doses to give the same fraction of surviving bacteria (Fig. 3). Proteins $\beta$ and $\delta$, in contrast, showed a pattern of induction quite different for the two types of inducing radiation. Despite the considerable scatter of points for various doses (Fig. 2 and 3A), $\beta$ was, in all samples of UV-treated cells, the most abundantly synthesized protein, whereas it was only moderately induced by electron irradiation (Fig. 3B). A shift in quite the opposite direction was found for $\delta$, which in electron-irradiated cells grew to be the most prominent protein seen on the gel (Fig. 1D) and accounted for 1% of total methionine incorporated (Fig. 3B).

To induce damage by a chemical mutagen rather than by irradiation, a culture of *M. radiodurans* was exposed to 10 $\mu$g of mitomycin C per ml in the growth medium for 20 min and subsequently labeled with [$^{35}$S]methionine for 30 min. Mitomycin C reduced the viable cell

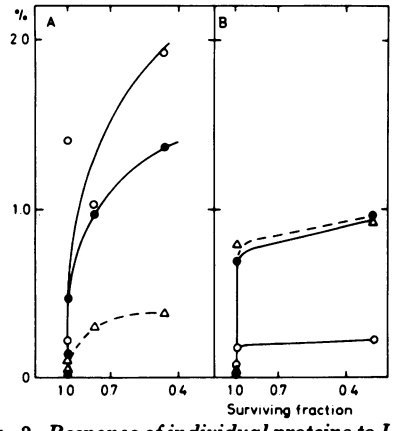


FIG. 3. *Response of individual proteins to UV and ionizing radiation. The percentage of [$^{35}$S]methionine incorporated into α (●), β (○), and δ (△) have been plotted for doses leaving 30 to 100% surviving cells. The induction of protein γ was very similar in UV- and electron-irradiated cells and has been omitted for clarity. (A) UV-irradiated cells. (B) Electron-irradiated cells.*

count to 4% of the pretreatment number and at the same time induced proteins α (0.07% of total incorporation), β (0.49%), and δ (0.07%), whereas γ was not detectably above background.

Two different schemes were utilized to arrest DNA replication without directly introducing damage in the cellular DNA. First, nalidixic acid, which inhibits DNA replication in *M. radiodurans* (7), was added to 100 μg/ml, together with [$^{35}$S]methionine, and incubated for 30 min. No induction of the proteins α, β, γ, or δ was detectable, nor did the synthesis of any other protein increase conspicuously over that seen in untreated cells (data not shown). The same result was obtained with cells pretreated with nalidixic acid for 30 min before the initiation of labeling. Second, thymine starvation was effected in a thymine-requiring mutant of *M. radiodurans* strain MH 133 and in samples labeled either immediately after removal of thymine or after 1 or 2 h of thymine deprivation. Neither sample contained amounts of proteins α, β, γ, or δ measurably above basal level.

## DISCUSSION

**Induced proteins and DNA repair.** Induced synthesis of the four proteins α, β, γ, and δ appears to occur specifically under conditions where the cellular DNA has been damaged. First, interference with DNA replication does not, per se, induce synthesis of the proteins. Nalidixic acid and thymine starvation, which both prevent replication, failed to elicit synthesis of either protein. Second, induction is not a mere result of cell death since even sublethal doses appreciably stimulated the synthesis of α, β, γ, and δ.

Continued protein synthesis after irradiation of *M. radiodurans* is necessary not only for maximal cell survival (6, 22), but also more specifically needed for rejoining of DNA strands broken by irradiation (6, 19). Furthermore, inhibition of protein synthesis causes excessive postirradiation degradation of cellular DNA (6). It is tempting to associate the greatly enhanced synthesis of the specific proteins α, β, γ, and δ with the need for protein synthesis in the period subsequent to irradiation and thus to attribute to these proteins a role in the processes of DNA repair. No direct evidence for their participation in DNA repair has been obtained, however, and it cannot be ruled out that other proteins synthesized after irradiation could excel α, β, γ, and δ in activity if not in abundance.

One clue to the action of the four proteins induced in irradiated cells may be the very quantities in which they are produced. Within the sublethal dose range of UV light, the degree of induction for each of the proteins is roughly linear with the dose (Fig. 2). For the most abundant protein, β, the differential rate of synthesis is some 1.5%/min of irradiation given to the cells. Assuming the methionine content to be similar in the induced proteins and the average protein of *M. radiodurans*, one can calculate the amount of β from the protein to DNA ratio of 10 in *M. radiodurans* (9), a cellular DNA content of $1.3 \times 10^{10}$ daltons (12), the differential rate of synthesis of 0.015 per min of irradiation, and a labeling period of 30 min out of a generation time of 160 min. For cultures exposed to 0.5 min of radiation, a dose well within the sublethal range, each cell is then calculated to contain $(2^{30/160} - 1) \times 10 \times 1.3 \times 10^{10} \times 0.015 \times 0.5 = 1.4 \times 10^{10}$ daltons of β after 30 min of postirradiation incubation. With a molecular weight for β of 29,000 (Table 1), this amounts to 4,800 molecules of β per cell. Thirty seconds of UV irradiation at the flux used (2 W/m$^2$) produces about 4,500 thymine dimers per cell of *M. radiodurans* (27). Considering the uncertainties involved and the assumptions made, particularly on the methionine content of β, not too much emphasis should be placed on the close agreement between the numbers calculated for protein β and for thymine dimers. However, the great abundance of α, β, γ, and δ certainly does suggest a stoichiometric mode of action rather than a catalytic one. Protein X in *E. coli* shows affinity for single-stranded DNA (13) and has been proposed to protect such DNA from nucleolytic attack (11, 34, 40). The proteins in *M. radiodurans* could fulfill a similar function by interacting stoichiometrically with variously damaged stretches of

of the DNA. It may be of significance that at the same doses where the linear relationship between dose and rate of synthesis of $\alpha$, $\beta$, $\gamma$, and $\delta$ breaks down, the cells appear unable to repair all the damage inflicted and the number of viable cells starts to decline (Fig. 2 and 3).

**Signal leading to induction.** The data obtained with UV light and ionizing radiation (Fig. 3) indicate a differential pattern of induction in *M. radiodurans* in response to various injurious agents. The prevailing photoproduct after UV irradiation is intrastrand thymine dimers (29), whereas the damages produced by ionizing radiation are much more diverse including both base damages and strand scissions (4, 14, 18). The local helix distortions caused by thymine dimers seem sufficiently distinctive from the strand breaks, which by themselves cause relaxation of greater domains of the chromosome (42), to enable the cell to differentiate its response.

One model, speculative but attractive for its simplicity and directness, which is consistent with the experimental data would postulate the induced proteins to be of the autorepressor type controlling their own synthesis (36). In addition, e.g., $\beta$ would possess affinity for thymine dimers or more generally for stretches of DNA with local helix distortions, whereas $\delta$ would bind to relaxed domains. Under inducing conditions the damage specific protein would be soaked up by the lesions, removed from its own operator, and synthesized until the damages had been titrated out. In the real world, a control system based on this principle has been demonstrated for phage $\lambda$ where the $\lambda$ repressor protein can be removed from $\lambda$ operators by radiation-induced sites in the host DNA with affinity for the repressor (37). The alternative hypothesis that general DNA degradation products constitute the inducing signal (11) would seem insufficient to explain the differentiated response in *M. radiodurans*. Evidence against this hypothesis has emerged even for the uniform induction of protein X seen for a number of damaging agents in *E. coli* (21).

Nalidixic acid and thymine starvation failed to induce $\alpha$, $\beta$, $\gamma$, and $\delta$ in *M. radiodurans*. Although inhibiting DNA replication, these treatments are not known to introduce any primary DNA damage and distortion of the DNA helix. Neither does nalidixic acid cause significant DNA degradation over several hours of treatment in *M. radiodurans* (7). Thus, induction of the radiation-inducible proteins of *M. radiodurans* by nalidixic acid might not be expected whether degradation products or changes in DNA structure constitute the triggering signal. Clarification of the mechanisms, both of the induction and the action of these proteins, will be greatly facilitated by experiments with suitable mutant strains altered in their repair capabilities.

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