# REDUCED DNA REPAIR DURING DIFFERENTIATION OF A MYOGENIC CELL LINE

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ABSTRACT

Repair synthesis induced by 4-nitroquinoline-1-oxide (4NQO) in L6 myoblasts before and after cellular fusion was measured by [$^3$H]thymidine incorporation into unreplicated DNA. The level of repair synthesis was reduced after the cells had fused into myotubes. The terminal addition of radioactive nucleotides into DNA strands occurred only to a minor extent, and the dilution of [$^3$H]thymidine by intracellular nucleotide pools was shown not to be responsible for the observed difference in repair synthesis. Both the initial rate and the overall incorporation of [$^3$H]thymidine were found to be 50% lower in the myotubes.

4NQO treatment of myoblasts and myotubes induced modifications in the DNA which were observed as single-strand breaks during alkaline sucrose sedimentation. After the myoblasts were allowed a post-treatment incubation, most of the single-strand breaks were no longer apparent. In contrast, a post-treatment incubation of myotubes did not change the extent of single-strand breakage seen. Both myoblasts and myotubes were equally effective in repairing single-strand breaks induced by X radiation. It would appear that when myoblasts fuse, a repair enzyme activity is lost, probably an endonuclease that recognizes one of the 4 NQO modifications of DNA. The result observed is a partial loss of repair synthetic ability and a complete loss of ability to remove the modification that appears as a single-strand break in alkali.

During the differentiation of muscle cells, a number of enzyme activities rise and others fall (8). In the latter category is an enzyme or enzymes associated with the repair of DNA. Thus, Hahn et al. (4) reported that methyl methanesulfonate-stimulated unscheduled DNA synthesis was readily apparent in freshly cultured rat embryo myoblasts, but this activity declined considerably as the myoblasts fused. Stockdale (10) compared ultraviolet light-stimulated unscheduled DNA synthesis in cloned chick embryo myoblasts and the multinucleated myotubes derived from them. The former were two to four times more active. In a subsequent paper, Stockdale and O'Neill (11) showed that the unscheduled DNA synthesis was due to repair synthesis. The isolation by Yaffe (15) of $L_6$ cells, an established line of myoblasts which has retained the ability to fuse and form myotubes, has provided a promising system for studying the differentiation of muscle cells. These cells display the same characteristic changes in biochemistry as freshly explanted embryonic muscle cells in culture, (8) but are more amenable to experimental manipulation. We have therefore chosen to examine the ability of cultured $L_6$ muscle cells, in the undifferentiated myoblast form and the differentiated myotube form, to repair DNA. Repair synthesis was measured by the incorporation of

[$^3$H]thymidine into unreplicated DNA, and the repair of DNA strand breaks was examined by sedimentation in an alkaline sucrose gradient. To induce repair, we used 4-nitroquinoline-1-oxide (4 NQO). This compound is mutagenic and carcinogenic, and its effect on biological systems is similar to that of ultraviolet light (9, 12).

## MATERIALS AND METHODS

### *Cell Cultures*

The $L_6$ line of rat myoblast was carried as a monolayer culture in Dulbecco modified Eagle's medium supplemented by 10% horse serum and gentamycin (50 μg/ml). Cells were subcultured at $1 \times 10^6$ per culture flask (75 cm$^2$; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) or at $1 \times 10^5$ per petri dish (60 × 10 mm, Falcon), and 2- to 3-day old cultures were used as myoblasts. On day 6, when fusion was well in progress, the cultures were treated with $10^{-7}$ M FdUrd for 24 h whereby nonfused myoblasts became detached and were removed (1). The remaining myotubes were used on day 7. All experiments were done in Eagle's minimal essential medium supplemented by 10% fetal calf serum and antibiotics; this second medium did not alter the growth of the cells.

### *Measurement of Repair Synthesis*

USING [$^3$H]THYMIDINE: The method used to measure repair synthesis was similar to that of Roberts et al. (7). Each flask of cells containing 10 ml of medium was incubated at 37°C with FdUrd ($10^{-6}$ M) and BrdUrd (5 μg/ml) for 0.5 h, followed by $10^{-5}$ M 4NQO for 1 h. Controls received saline instead of 4NQO solution. The medium was then changed to one containing FdUrd and BrdUrd as before plus hydroxyurea ($10^{-2}$ M) and [$^3$H]thymidine (5 μCi/ml, sp act 21.2 Ci/mmol). The effect of using [$^3$H]thymidine and BrdUrd simultaneously is equivalent to that of using [$^3$H]BrdUrd, and incorporation of $^3$H would essentially indicate substitution by BrdUrd. Hydroxyurea was present to suppress semiconservative DNA synthesis, and the protocol was designed to minimize terminal labeling of incomplete DNA fragments. The incorporation of BrdUrd into replicating DNA increases the buoyant density of the molecule which, then, is distinguishable from unreplicated DNA in a CsCl gradient. Since repair synthesis involves the incorporation of only very small amounts of nucleotide precursors, incorporation of BrdUrd during repair is not sufficient to alter significantly the buoyant density of the DNA (2). In other words, radioactivity recovered in DNA of normal density would indicate the repair phenomenon.

The method for isolating DNA was essentially that of Flamm et al. (3). Two identically treated flask cultures were pooled for each experiment. After a 3-h labeling period, the cells were washed, scraped off, and resuspended in 0.5 ml of a solution containing 80 mM EDTA and 20 mM NaCl. Then, 5 ml of the same solution containing, in addition, 1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) was added, and the samples were kept in ice for 10 min. These broken cell preparations were centrifuged at 1,000 *g* for 10 min, and the nuclei in the pellet were lysed in 0.4 ml of 1 mM Tris-HCl, 0.5 mM EDTA, 0.5% sodium dodecyl sulfate (SDS) (pH 7.6). This lysate was mixed with 4 ml of CsCl solution (1.44 g CsCl per ml of buffer containing 10 mM Tris-HCl, 1 mM EDTA, pH 8, $\rho$ = 1.77 g/cm$^3$), and centrifuged at 10,000 rpm for 30 min. The protein that precipitated from the lysate formed a thin layer at the top of the tube. 4.0 ml of the clear solution ($\rho$ = 1.71 g/cm$^3$) was carefully transferred into a cellulose nitrate tube, covered with oil, and centrifuged at 45,000 rpm and 20°C for 24 h. Fractions measuring 0.25 ml were collected and, on some of these, refractive indices were measured in order to determine the density. The fractions were then diluted to 1 ml with water, and their optical densities were read at 260 nm. A 0.2-ml sample of each diluted fraction was mixed with 10 ml of Aquasol (New England Nuclear, Boston, Mass.) for liquid scintillation counting.

USING [$^{32}$P]ORTHOPHOSPHATE: The measurement of repair synthesis was carried out exactly as described above except that $^{32}$P (carrier-free, 20 μCi/ml of medium) and [$^3$H]thymidine (10 μCi/ml) were used for nucleotide labeling during the post-treatment incubation period. After centrifugation in CsCl, the parental DNA fractions ($\rho$ = 1.70–1.71 g/cm$^3$) from myoblasts and myotubes were dialysed against a solution containing 0.0015 M sodium citrate, 0.015 M NaCl, and then mixed with 2 ml of a buffer containing 0.08 M $Na_2HPO_4$, 0.11 N NaOH (pH 12.5), and enough distilled water to give a final vol of 4.5 ml. These preparations were added to 6.5 g CsCl, and the solutions were centrifuged in a Ti50 rotor at 37,000 rpm and 20°C for 36 h. Fractions of 0.35 ml were collected, and each was neutralized by 0.7 ml of 0.005 N HCl. Optical densities were measured at 260 nm. An equal vol of 10% trichloroacetic acid was added to the fractions. The precipitate was caught on fiber glass filters before liquid scintillation counting.

### *Phosphodiesterase Digestion*

After centrifugation in CsCl, the fractions containing parental DNA ($\rho$ = 1.70–1.71 g/cm$^3$) of the 4NQO-treated culture were pooled and dialyzed. The sample was mixed with buffer such that the final preparation contained 30 mM Tris-HCl, 70 mM $MgCl_2$ (pH 8.5). It was then mixed with purified DNA($^{32}$P) and heated for 15 min, at 90°C. The DNA($^{32}$P) was isolated from BHK cells which had been grown for 24 h in medium containing [$^{32}$P]orthophosphate (0.2 μCi carrier-free radiophosphate added per ml of medium). Venom phosphodiester-

ase (Worthington Biochemical Corp., Freehold, N.J.) was dissolved in the above-mentioned buffer and added to the DNA to a final concentration of 20 μg/ml. The reaction mixture was incubated at 37°C. Portions measuring 0.5 ml were removed at intervals and mixed with an equal volume of bovine serum albumin (1 mg/ml). After precipitation with trichloroacetic acid, radioactivity in the supernate was counted in Aquasol.

### *Incorporation of Thymidine into Total DNA*

Separate cultures in petri dishes were treated with $1 \times 10^{-5}$ M 4NQO or saline for 1 h before being incubated with [$^3$H]thymidine (10 μCi/ml) and hydroxyurea ($10^{-2}$ M) for 1, 3, and 8 h. The cells were collected by scraping, counted, and then frozen in 2 ml of a buffer containing 30 mM Tris-HCl, 70 mM $MgCl_2$ (pH 8.5). The number of myotube cells was taken to be equal to the number of myoblast cells measured in a replicate culture on the previous day. The cell suspension was thawed and bovine serum albumin was added to a final concentration of 1 mg/ml. An equal vol of 10% trichloroacetic acid was used for precipitation. After filtration, the filters (25 mm, Type A glass fiber, Gelman Instrument Co., Ann Arbor, Mich.) were counted in Aquasol.

### *Alkaline Sucrose Gradient Sedimentation Analysis*

2-day old cultures in petri dishes were incubated with [$^3$H]thymidine (0.5 μCi/ml) for 24 h, washed and replenished with fresh medium. They were used either immediately (myoblasts) or after fusion had occurred (myotubes). Single-strand breaks in DNA were produced by treating the cultures with $1 \times 10^{-5}$ M 4NQO for 1 h or by exposing them to 6 krad of gamma radiation. The latter was delivered by a Cobalt$^{60}$ Gamma Cell (Atomic Energy of Canada Ltd.) at a dose rate of 12 krad/min. The cell samples were analyzed then or after a post-treatment period of 3 or 5 h in fresh medium. Sedimentation analysis was carried out as previously described (13). Briefly, 4.7-ml sucrose gradients (5–20%) in a solution containing 0.3 M NaOH, 0.01% sodium dodecylsulfate, and 0.001 M EDTA were made by an ISCO 570 gradient former (Instrumentation Specialties Co., Lincoln, Neb.), and 0.3 ml of a lysing solution containing 0.5 M NaOH, 0.2% SDS and 0.01 M EDTA was layered on top of each gradient. Approximately $1 \times 10^4$ cells were delivered onto each gradient. The gradients were allowed to stand at room temperature for 10–12 h before they were centrifuged at 15,000 rpm and 20°C for 4.5 h in an SW 50.1 rotor. Fractions (0.2 ml) were collected from the top with an ISCO 640 fraction collector, and neutralized by HCl. Radioactivity in each fraction was then measured by liquid scintillation counting.

## RESULTS

The ability of the $L_6$ line of myoblasts and myotubes for repair synthesis of DNA was compared using the method of isopycnic centrifugation to separate the newly replicated DNA from the repaired but not replicated DNA. The profiles in Fig. 1 show the banding positions of the DNA in the CsCl gradients. The position of parental DNA is marked by the large peak of $OD_{260}$ absorbing material occurring at a buoyant density of 1.70–1.71 g/cm$^3$. Newly synthesized DNA, containing one bromouracil substituted strand and one ordinary strand, has a density of 1.73–1.74 g/cm$^3$ as indicated by the radioactivity in this region. The radioactivity associated with parental DNA is indicative of repair synthesis. The specific activity of this DNA was measured after the appropriate fractions were pooled and the CsCl removed by dialysis. In the control cultures not treated with 4 NQO (top panel), these values in cpm/μg DNA were 2.6 for the myoblasts and 8.8 for the myotubes. In the 4NQO-treated cultures (middle panel), the values were 112 and 66.5, respec-

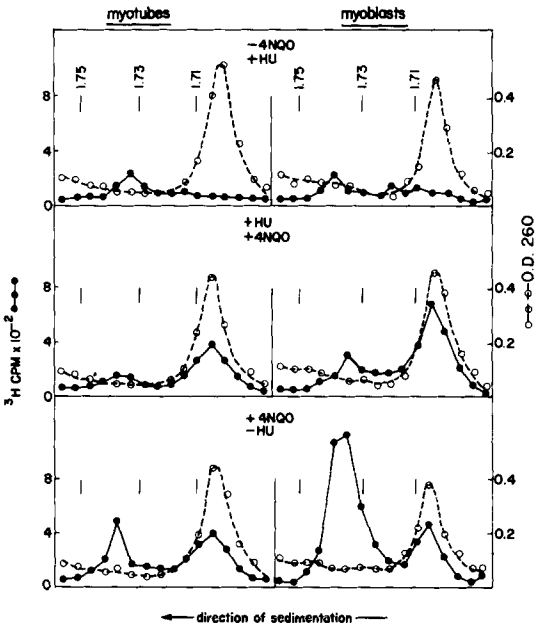


FIGURE 1 Isopycnic centrifugaton of DNA in neutral CsCl. Gradient profiles of DNA isolated from control cultures (top panel) and from treated cells (middle panel). In two samples (bottom panel), hydroxyurea was omitted during the incorporation period. Numbers and vertical bars in the diagram represent the densities of the corresponding fractions in g/cm$^3$.

tively. The myotubes exhibited about one-half the repair incorporation of the myoblasts. The small amount of incorporation in the controls was probably due to incomplete resolution of semiconservatively synthesized DNA, although hydroxyurea was used to inhibit normal DNA synthesis. Similar results were obtained without the use of hydroxyurea (lower panel), but, in this case, the fractions containing parental DNA had to be recentrifuged in alkaline CsCl in order to effect a sufficiently clean separation of newly synthesized DNA from parental DNA. The results shown in the lower panel of Fig. 1 also indicate that the myotube culture contained only a few dividing myoblasts because the amount of semiconservative synthesis in the myotube culture was small relative to that in the myoblast culture.

One explanation for the lower specific activity obtained with the myotubes was that the labeled thymidine was diluted by a larger nucleotide pool in the myotube. This possibility was checked by following the repair process with an additional label, $^{32}P$. Assuming that $^{32}P$ labeled all four nucleotide triphosphates uniformly, the $^{3}H$:$^{32}P$ radioactivity recovered in the repaired DNA would reflect the dilution of [$^{3}H$]thymidine by the intracellular nucleotide pool. When such an experiment was done (see Materials and Methods section), the $^{3}H$:$^{32}P$ ratios for myoblasts and myotubes were found to be 2.69 and 2.26, respectively. That is, the dilution of [$^{3}H$]thymidine, by itself, cannot account for the difference in repair activity observed.

An error in our measurement, however, could result from the presence of replicating DNA molecules which have not completed making a new strand at the time of addition of [$^{3}H$]thymidine. End-addition of radioactive nucleotides to preexisting DNA would give exaggerated values for repair synthesis. Although the probability that the parental DNA fraction did contain some of these molecules was reduced by the incubation with BrdUrd before 4NQO treatment, possible contamination must be evaluated. Therefore, the rate at which radioactive nucleotides were released from parental DNA by snake venom phosphodiesterase was examined. Parental DNA fractions obtained from the 4NQO-treated cultures were denatured by heat before digestion. For comparison, the digestion mixture also contained DNA that was uniformly labeled with $^{32}P$. Fig. 2 shows the time-course of release of acid-soluble radioactivity. Clearly, the nucleotides incorporated by the 4 NQO-treated cells were not more susceptible to the exonucleolytic attack than those in uniformly labeled DNA($^{32}P$). This indicates that the labeled nucleotides incorporated after 4NQO treatment were located internally rather than at the ends of DNA strands. Thus, the repair activity in the myoblasts is unlikely an artefact effected by end-addition.

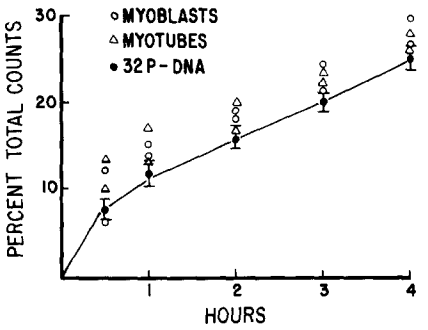


FIGURE 2 Release of acid-soluble radioactivity after phosphodiesterase digestion of uniformly labeled DNA($^{32}P$), and repaired DNA($^{3}H$) from myoblasts and myotubes. Treatment of cells and digestion by phosphodiesterase were as described in the Materials and Methods section. Duplicated samples were used for repaired DNA. The curve obtained with DNA-($^{32}P$) represents the mean of four samples, with variations included in the error bars. Total $^{32}P$ per sample is 3,500 cpm; total $^{3}H$ per sample is 400-800 cpm.

The preceding results indicate that the initial rate of repair synthesis in myotubes was about one-half of that in myoblasts, but it seemed possible that in myotubes the repair process might persist for a longer period of time and eventually give the same total extent of repair. This possibility was investigated through a study of the kinetics of incorporation of [$^{3}H$]thymidine into the DNA of 4NQO-treated myotubes and myoblasts in the presence of hydroxyurea (Fig. 3). The use of hydroxyurea has effectively inhibited semiconservative DNA synthesis as judged by the slight incorporation of [$^{3}H$]thymidine in the control cells. Treatment with 4NQO has stimulated a considerable further incoporation. The previous results indicated that this incorporation was due to repair synthesis. It is apparent from the results shown in Fig. 3 that the initial rate of repair synthesis was greater in the myoblasts. In addition, at the end of an 8-h post-treatment incubation, when the incorporation was reaching a plateau, the response to 4

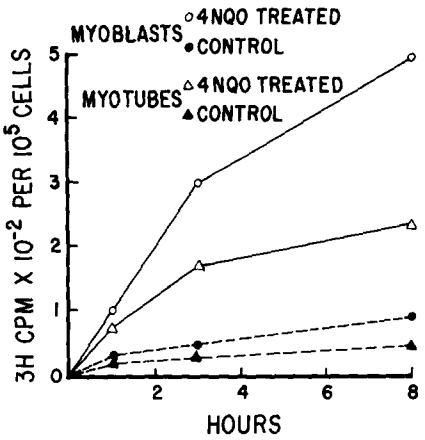


FIGURE 3 Kinetics of 4NQO-stimulated incorporation of [$^3$H]thymidine into DNA of myoblasts and myotubes in the presence of hydroxyurea. The number of cells in the fused cultures was estimated separately from confluent plates just before fusion occurred. Each point in the figure is the average of results obtained from duplicated samples.

NQO by the myotubes remained at about 50% of that of the myoblasts. The fusion of myoblasts has led to a reduction in both the rate and the total amount of repair synthesis.

As a further means of characterizing the DNA damage and repair process in myoblasts and myotubes, we employed the technique of sedimentation in an alkaline sucrose gradient. We had previously shown with this technique (14) that 4NQO treatment of HeLa and L cells led to the appearance of single-strand breaks in the DNA from these cells. This damage was seen to be repaired by HeLa but not L cells. The sedimentation patterns for the DNA from the 4NQO-treated myoblasts and myotubes are shown in Fig. 4. The effect of the 4NQO treatment was the same for both cell types, namely, a reduction in the sedimentation coefficient of the DNA from a control value of approximately 160 S to a value of approximately 30 S. When the cells were incubated for 3 h in fresh medium after the 4NQO treatment, and then layered onto the gradient for lysis and centrifugation, the DNA of the myoblasts had increased in size, whereas that of the myotubes remained unchanged. An additional 2-h post-treatment incubation produced no further change in either case. Thus, after myoblasts have fused, there is a complete loss of the ability to repair a form of damage that is seen as a single-strand break in alkali and yet there is only partial loss of ability for repair synthesis. One explanation of these findings is that the repair synthesis observed is incomplete and the rejoining step is left undone by the myotubes. But this is unlikely since the internal location of the nucleotides incorporated during repair (Fig. 2) disproves the presence of open-ended repaired regions in the DNA. Rather, it seems that the nature of the breaks induced by 4NQO is the underlying cause of our observation. That a further 2-h post-treatment incubation of myoblasts did not alter the sizes of the DNA indicates that some breaks were never rejoined.

To further investigate the rejoining step, we examined X-irradiated cells. X rays can directly induce single-strand breaks which are thought to be repaired by the insertion of only one or a few bases per lesion followed by rejoining (5, 6). The use of X rays then, in effect, allows the rejoining step to be viewed by itself. The sedimentation patterns in Fig. 5 were obtained with X-irradiated myoblasts and myotubes before and after a 5-h recovery period. A decrease in the size of the DNA after irradiation signalled the induction of

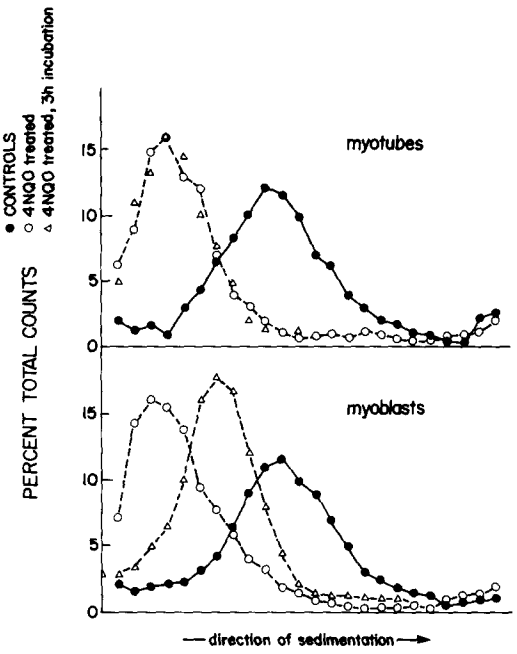


FIGURE 4 Sedimentation patterns of DNA in alkaline sucrose gradients after 4NQO treatment. The 4NQO ($1 \times 10^{-5}$ M) treatment of prelabeled cells was carried out for 1 h, and medium was replaced for further incubations. The control received no 4NQO. Each gradient contained $10\text{-}14 \times 10^3$ cpm.

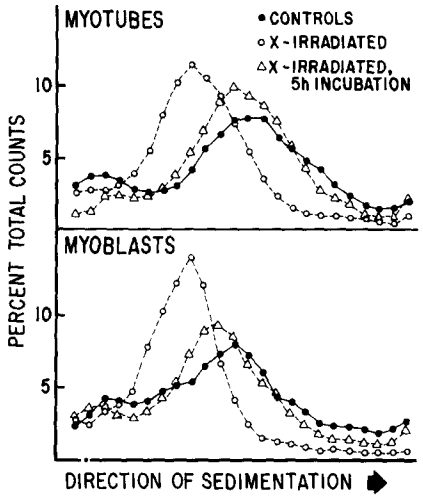


FIGURE 5 Repair of X-ray-induced breaks. Prelabeled cells were irradiated with a dose of 6 krad and their DNA was analyzed before and after a 5-h recovery in fresh medium. The control cultures were not irradiated. Each gradient contained 10–15 × $10^3$ cpm.

breaks. These were rejoined after post-treatment recovery, leading to a restitution of the size of DNA comparable to that of the control cells. More important is that the myotubes are also able to repair X-ray-induced breaks. It appears that they do possess the enzyme or cofactor required for rejoining. This observation, besides providing additional evidence against an incomplete repair process, further implies that the lack of rejoining of 4NQO-induced breaks is likely a consequence of some peculiarity associated with 4NQO.

## DISCUSSION

This study has shown that the permanent line of myoblasts, $L_6$, behaves like primary myoblast cells in its loss of capacity for repair synthesis after fusion to myotubes has occurred. This loss represented a reduction in both the rate and the extent of repair synthesis. Furthermore, the fused cells appeared no longer able to rejoin single-strand breaks induced in their DNA by 4NQO, although they were still capable of rejoining single-strand breaks induced by X irradiation. It should be recalled that the action of 4NQO on mammalian cells mimics that of UV-light rather than X irradiation (9), and it is likely therefore that 4NQO does not induce the formation of single-strand breaks in vivo. Rather, the breaks seen are the result of the action of alkali on the 4NQO-modified DNA. Our results can be explained as follows. 4NQO induces at least two kinds of modification in DNA which can be removed by an enzymatic repair process. When the modifications are not removed, at least one shows up as a single-strand break during alkaline sucrose sedimentation. The two modifications are recognized by separate endonucleases. Myoblasts contain both, but myotubes contain only one. Thus, in the myotubes repair synthesis is reduced, and the unrepaired modification is observed as a persisting single-strand break in alkali. 4NQO also induces a third modification which is observed as an irreparable strand break in alkali. Its presence accounts for the partial rejoining seen in the myoblasts. A scheme of this model is shown in Fig. 6.

In previous studies on differentiating systems, the observed reduction in repair activity has been linked to a loss in some enzyme activity (4, 11), but the possibility of a pool size effect was not eliminated. Presently, we have shown that our observation is not due to an altered nucleotide pool in the myotubes. Thus, the loss of enzyme activity has become an even more reasonable explanation. Definite proof, however, must await the identification and purification of the responsible enzyme or enzymes.

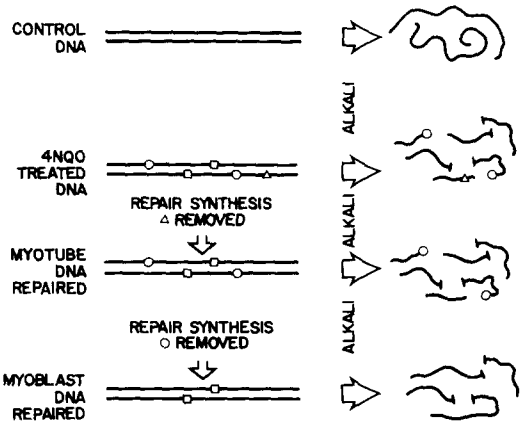


FIGURE 6 Schematic representation of 4NQO damage and its repair. 4NQO causes three kinds of modifications in DNA: the first modification (△) is repaired by the cell before and after fusion, and is apparently alkali-resistant. The second modificaton (○) is not repaired by the myotubes probably because of the loss of an enzyme. The unremoved damage leads to strand breakage in alkali. The third modification (□) is irreparable and alkali labile.

We are grateful to Dr. R. Sridhar for providing us with his X-ray data on $L_6$ myoblasts and myotubes.

This work was supported by the Medical Research Council of Canada.

*Received for publication 15 March 1976, and in revised form 6 May 1976.*

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