Enzymatic mechanisms for the repair of UV irradiated DNA

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Irradiation of DNA with ultraviolet light (UV) results in the formation of two general types of photoproducts of pyrimidines. Either dimers are formed between adjacent pyrimidine nucleotides (thymine-thymine, TpT : cytosine-cytosine, CpC or cytosine-thymine, CpT) by cyclobutane ring formation at the 5,6-double bond sites, or the elements of water may add across the same sites of cytosine to form a water adduct (photohydrate). The latter short-lived photo-product is most likely responsible for the transitions observed (ONO, WILSON & GROSSMAN, 1965), whereas the former type of photoproducts lead to the death of the cells (SETLOW & SETLOW, 1962 ; 1963 ; WALKER, 1963).

There are two general mechanisms by which cells may reverse the adverse effects of such irradiation. Either the photoproducts may be directly repaired by reversal, for example of the dimer back to the monomer, through visible light photoreactivation (RUPERT, 1960), or the damage may be removed from the DNA - generally in the absence of visible light (SETLOW & CARRIER, 1964). The latter dark repair process seems to be a more general biological mechanism and appears to be related to other general functions of the cells such as recombination (HOWARD-FLANDERS & BOYCE, 1966) or replication (KELLY *et al.*, 1969).

Dark repair is not confined to UV-induced damage, but also includes lesions produced by such diverse agents as mitomycin, X-rays, nitrous acid and certain mono- and bifunctional alkylating agents (HOWARD-FLANDERS & BOYCE, 1966). Therefore, enzymes involved in the initial stages of repair system are not necessarily specific for the primary chemical changes in the damaged DNA, but may recognize the secondary consequences of UV; for example localized regions of distortion caused by dimer formation.

Our laboratory has been concerned with the isolation of enzymes which can recognize localized regions of distortion in damaged DNA. *Micrococcus luteus*, a UV resistent organism, was chosen as the bacterial source

because of its low endogenous nuclease levels, which permit the detection of small amounts of distortion specific endo- and exonucleases. This paper describes the properties of two enzymes which together excise photoproducts from UV irradiated double stranded DNA. The first enzyme is an endonuclease that causes a single phosphodiester bond break 5'- to and imminent to each pyrimidine dimer thus creating a single stranded region with a 5' -hydroxyl end. This incision step is followed by excision of the damaged region by a second specific enzyme, an exonuclease which is capable of degrading the exposed single stranded region arising from the incision step. The combined action of the two enzymes results in the release of acid-soluble nucleotides containing thymine-thymine dimers.

Evidence supporting the hypothesis of single-strand incision by the UV -endonuclease rather than double-strand cleavage is presented in Fig. 1.

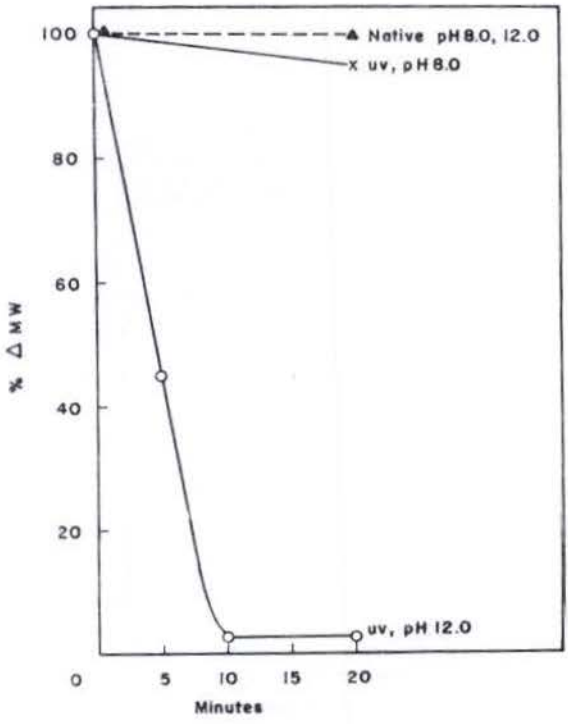


Fig. 1. — Change in the sedimentation properties of UV irradiated DNA as a function of UV endonucleolytic incision.

If DNA treated with the enzyme is subjected to ultracentrifugation at neutral and alkaline pH's, a differentiation between single- and double-strand breaks can be made. The change in the sedimentation coefficient only at alkaline pH's indicates single-stranded scission.

The assay for the endonuclease is based on its ability to cause single-strand breaks by hydrolyzing phosphodiester bonds in UV -irradiated double-stranded ^{32}P-DNA. By measuring the appearance of ^{32}P-labeled phosphomonoester groups using bacterial alkaline phosphatase, one can determine the number of phosphodiester bonds broken. Incision by the endonuclease produces a single- stranded region which is subsequently recognized by the UV -exonuclease. Actual nucleotide release requires the presence of both enzymes.

Purification of the UV endonuclease

The incising enzyme has been purified considerably according to the general methods given in Table 1. This preparation (fraction IV) has no detectable conflicting enzymes such as exonucleases or phosphomonoesterases.

TABLE 1.

Purification of UV-endonuclease

Fraction	Specific Activity *	Total units
1. Lysis and sonication	32.5	794,200
2. Phase separation	146	832,950
3. Phosphocellulose	10,212	323,300
4. Diaflo concentration	10,588	190,800
5. G-75 and DNA-cellulose	165,000	19,250

* Specific activity is in units per mg protein.

The absolute dependency on UV irradiation for activity also points to the absence of non-specific endonucleases (Fig. 2).

It has been possible to resolve the endonuclease almost entirely from contaminating exonuclease activity. Under certain purification conditions, activation by Mg^{++} and certain monovalent cations has been observed. The pH optimum is between 6.5 and 7.5. Fig. 2 shows that the endonuclease requires UV -irradiated double-stranded DNA and that its activity is dose dependent.

The high efficiency of the endonuclease for recognizing photoproduct induced distortions is indicated also in Fig. 2. In the region between 10^4

and 10^5 ergs/mm^2 at 280 nm where thymine-thymine dimer levels have been determined independently by chromatographic methods, one phosphodiester bond is broken for each dimer. According to the work of SETLOW &

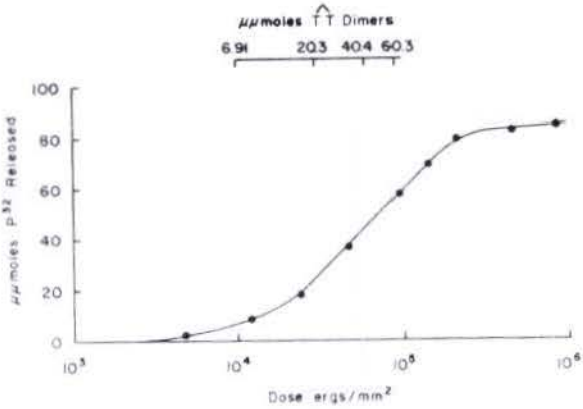


Fig. 2. — Dose dependency requirement for the UV endonuclease.

CARRIER (1966), approximately 94 % of all pyrimidine-pyrimidine dimers at 280 nm contains at least one thymine residue. Therefore, the enzyme not only exhibits a specificity for UV - induced distorted regions, but also quantitatively produces a single incision at each of these areas. Preliminary data indicate that enzymatic attack occurs on the strand containing the photoproducts rather than on the complementary strand.

Purification of the UV exonuclease

The purification of the excising enzyme has been accomplished by the techniques outlined in Table 2. This enzyme too is free of conflicting nucleases, phosphomonoesterases and particularly the DNA polymerase. The significance of this enzymatic resolution will be discussed in later sections of this article.

The exonuclease is assayed by measuring the conversion of UV -irradiated ^{32}P -denatured DNA into acid-soluble nucleotides. During purification a comparison is made between irradiated and unirradiated denatured DNA as substrate. A time course of the enzymatic reaction demonstrates that the purified UV -exonuclease does not discriminate between denatured ^{32}P -DNA and irradiated denatured ^{32}P -DNA (Fig. 3). However, in *M. luteus* a second exonuclease is present which actively hydrolyzes denatured DNA but is inhibited by irradiated denatured DNA.

TABLE 2.

Purification of UV-exonuclease

Fraction	Specific Activity *	Total units
1. Lysis and sonication	6.2	29,550
2. Streptomycin	9.4	27,700
3. Ammonium sulfate (70 %)	12.0	26,299
4. DE-52 cellulose	96.0	13,493
5. DNA-cellulose	6,720.0	1,650 6,820 **

* Specific activity is in units per mg protein.

** The yield in this step is dependent on the batch and age of the DNA-cellulose resin. Reproducibility is poor.

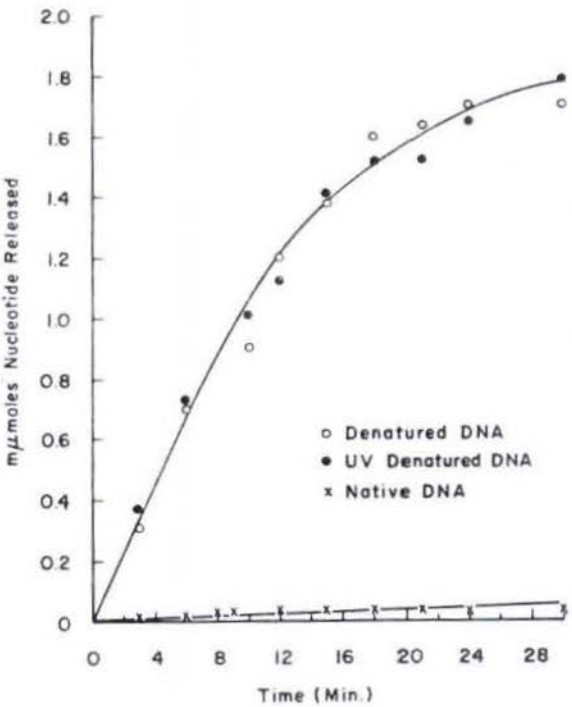


Fig. 3. — Substrate specificity of the UV endonuclease.

In addition, the UV -exonuclease is able to degrade 5'-0-phosphoryloligothymidylates of various chain lenghts (see Table 3). In contrast to exo-

TABLE 3.

Hydrolysis of oligonucleotides by UV-exonuclease

	Pentamer count/min	Tetramer count/min	Trimer count/min		Dimer count/min	Monomer count/min	Total count/min	Rf
1. Monomer . .	—	—	—		—	—	—	0.656
2. Dimer . . .								0.591
— enzyme .	—	—	—		2926	242	3168	
+ enzyme .	—	—	—		202	2329	2531	
3. Trimer . . .								0.451
— enzyme .	—	—	2936		274	68	2677	
+ enzyme .	—	—	852		1248	691	2791	
4. Tetramer . .								0.355
— enzyme .	—	2616		309		50	2975	
+ enzyme .	—	589		1721		714	3032	
5. Pentamer . .								0.258
— enzyme .	1834					79	2080	
+ enzyme .	443					1148	2393	

Thymidine 0.836

nuclease I from *Escherichia coli*, this enzyme can completely hydrolyze 5′-0-phosphorylthymidylthymidine. These oligonucleotides have not been irradiated.

The number of photoproducts in denatured DNA produced at 280 nm does not effect UV -exonuclease activity (Fig. 4). In contrast, snake venom pho-

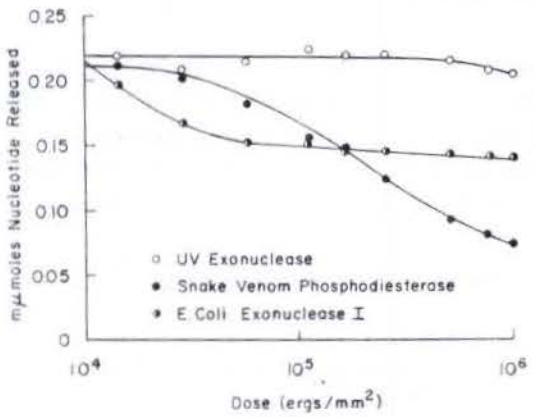


Fig. 4. — Effect of UV dose on the activity of the UV exonuclease.

sphodiesterase, a similar enzyme is markedly inhibited by irradiated DNA. The fact that UV -exonuclease activity is not depressed by a photoproduct

containing DNA strongly indicates that this enzyme is involved in the dark repair system.

Nucleotide release from both denatured and irradiated denatured DNA occurs at the same rate and levels off after two hours with 100 per cent hydrolysis (Table 4). The degradation of native DNA, irradiated native DNA,

TABLE 4.

Specificity of UV-exonuclease

Compound	Amount of substrate (mμmoles)	Nucleotide release (mμmoles)
1. Irradiated heat-denatured *E. coli* DNA	2.4	2.16
2. Heat-denatured DNA	2.4	2.16
3. Irradiated native DNA	2.4	0.025
4. Native DNA	2.4	0.030
5. RNA	2.4	0.010
6. Irradiated native DNA (UV-endonuclease treated)	7.58 (0.058 phosphodiester bonds broken)	0.30 *

* Native irradiated DNA was treated with the UV-endonuclease until 0.058 mμmoles of phosphodiester bonds had been broken. Nucleotide release in this case is reported for a 4-hr period. The other compounds were incubated for a 2-hr period under standard assay conditions.

or RNA amounts to no more than 1 per cent. However, native irradiated DNA which has been preincubated with UV — endonuclease so that 0.058 mμmoles of phosphodiester bonds have been broken, is susceptible to the UV-exonuclease. Also, oligonucleotides, including dinucleotides, are hydrolyzed by this enzyme yielding 5'-mononucleotides as the major products released.

The combined action of the UV endo- and exonuclease

If UV-exonuclease is allowed to react with UV-irradiated double-stranded ^{32}P-DNA (1.0×10^2 ergs/mm^2), which has been previously treated with UV-endonuclease, until the amount of nucleotide released has reached a maximum limit, an estimate of the size of the excised region can be determined. From Fig. 5 it is clear that five nucleotides are excised for every single strand break. This is important because previous work employing ^{3}H-thymine labeled

DNA indicated that three thymine residues are present in each dimer region (GROSSMAN *et al.*, 1968). Thus, dimer formation appears more likely to occur in the thymine-rich areas.

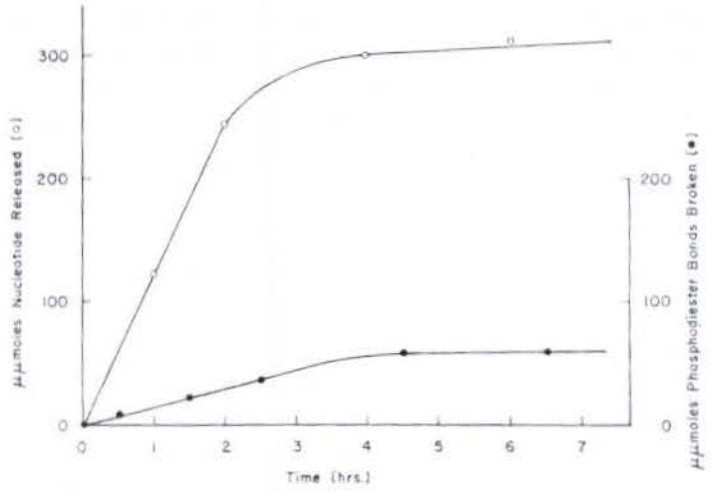


Fig. 5. — Relationship between total nucleotide released after excision by the combined action of the UV exo- and endonucleases as compared to the number of phosphodiester bonds broken by the UV endonuclease during the incision process.

TABLE 5.

Excision of pyrimidine dimers from UV-irradiated ^{3}H-DNA

	Acid-soluble			Acid-precipitable				
	Thymine dimer (μμmoles)	Thymine (μμmoles)	$\widehat{TT}$/T	Thymine dimer (μμmoles)	Δ	Thymine (μμmoles)	Δ	$\widehat{TT}$/T
Sample								
UV-irradiated ^{3}H-DNA no enzymes . .	0.0	0.0	—	118.2	—	1589	—	0.074
UV-irradiated ^{3}H-DNA UV-endo plus UV-exonuclease .	+ 78.4	+ 514.0	0.153	49.4	— 68.8	1053	— 536	0.047

A 2.5-hr incubation was carried out under conditions described in Fig. 2. The acid-soluble fraction was neutralized with 0.045 ml of 10 N KOH and the precipitate removed by centrifugation. The supernatant fluid was dried, resuspended in 0.1 ml H_2O and subjected to high-voltage electrophoresis. The acid-precipitable fraction was dissolved in 0.05 ml H_2O and 0.5 ml formic acid (97 %). After the solution had been hydrolyzed at 175°C for 2hr, it was analyzed by two-dimensional chromatography.

The digestion products released from UV-irradiated double stranded ^{3}H-DNA include mononucleotides as well as di- and trinucleotides containing the actual dimers. A study of the DNA (1.0×10^5 ergs/mm^2) before and after enzymatic treatment shows a 60 per cent decrease in the number of thymine dimers after excision (Table 5). Since it has been shown that the efficiency of dimer excision decreases with irradiation doses above 10^5 ergs/mm^2, a 100 per cent loss of thymine-containing dimers was not expected (Fig. 2). The UV-endonuclease preparation used in this experiment was slightly contaminated with exonuclease so that a more extensive degradation of DNA was observed than in Fig. 5.

Site of UV endonuclease action

The initial phosphodiester bond broken must be reasonably close to each thymine dimer pair such that excision of the dimer can occur accompanied by no more than three to four other nucleotides. This extremely efficient process occurs specifically 5'- to the (left side) location of the thymine dimer leaving a 3'-phosphoryl end on the undamaged region and a 5'-OH end on the damaged fragment. These findings are summarized in Fig. 6.

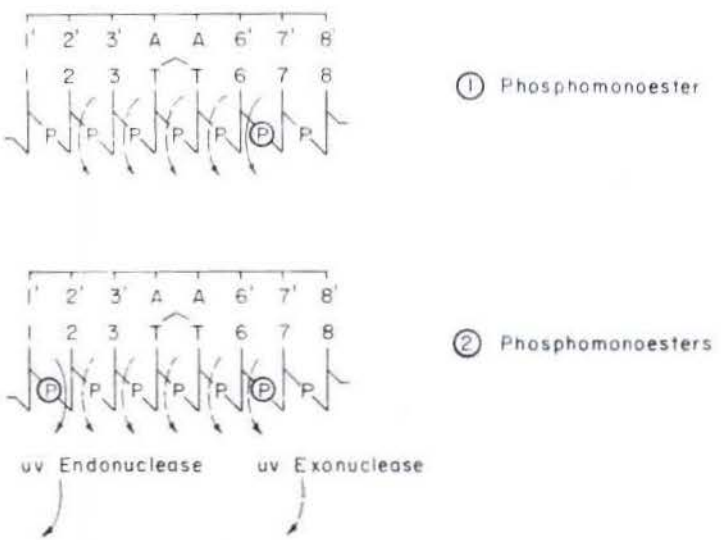


Fig. 6. — Possible sites of action during the incision by the UV endonuclease.

The evidence for such a general mechanism is based on two types of experimentation. The initial experimental results were based on the stoichiometry of the number of phosphodiester bonds formed on the polymer after the

incision step catalyzed by the endonuclease and the number of such groups appearing after the excision sequence when exposed to the exonuclease. After the incision step one phosphomonoester group is titratable by bacterial alkaline phosphomonoesterase (BAP) and a second appears after removal of the single stranded region by the UV exonuclease (Table 6).

TABLE 6.

Appearance of phosphomonoester groups in incised and excised DNA

Number of Phosphomonoester Groups after		
Time (mins)	Incision (pmoles)	Excision (pmoles)
90	22.3	43.6
150	36.2	68.9
270	58.0	112.0

The endonuclease can initiate hydrolysis either 5′ — or 3′ — to the dimer region. If the exonuclease initiates hydrolysis 3′- to the damage liberating only 5′ -mononucleotides an extra phosphomonoester group could not appear after excision (mechanism 1, Fig. 6). If the initial endonucleolytic break occurred 5′- to the damaged region it is to be expected that the exonuclease initiated hydrolysis from the 5′-end liberating 5′-monodeoxynucleotides (mechanism 2, Fig. 6). This is somewhat unusual for there is only one instance reported in which an exonuclease initiates hydrolysis at the 5′-terminus liberating 5′-mononucleotides. Normally such nucleotides are liberated when initiation occurs at the 3′-hydroxyl end of a chain. Conversely, 3′-mononucleotides are usually liberated when an exonuclease initiates hydrolysis at the 5′- end of a DNA chain. Therefore, a « symmetrical » type exonuclease activity would be required to satisfy the data that were obtained. As will be shown in the next section of this paper the UV exonuclease (excising enzyme) initiates hydrolysis from *either* the 5′ or 3′-terminus of single stranded DNA. Thus the UV endonuclease initiates hydrolysis 5′ - to the damaged region.

That the UV endonuclease leaves a 3′ –phosphoryl terminus on the undamaged fragment is provided from experimentation with the polynucleotide kinase. This enzyme in the presence of ATP specifically phosphorylates 5′- OH groups. Therefore, positive phosphorylation implies a free 5′-OH group. Pretreatment with bacterial alkaline phosphatase as a necessary first step for phoshorylation implies the presence of a 5′-phosphoryl group.

Such experiments were initiated with controls provided by the use of DNase treated ^{32}P-DNA. Phosphorylation was not observed even when the controls provided for quantitative phosphorylation with the kinase. It seemed likely that the presence of photoproducts might in fact have been inhibiting the action of the polynucleotide kinase. In order to examine this in more detail the incised irradiated ^{32}P-DNA was treated with purified photoreactivating enzyme from yeast which in the presence of visible light monomerizes thymine dimers. With the use of this enzyme (a gift of Dr. J. Setlow) and lacking phosphomonoesterase quantitative phosphorylation occurred with ^{32}P-ATP. Therefore the phosphate is left on the 3'- position to the left of 5'- to the damaged region (Table 7).

TABLE 7.

Requirements for phosphorylation of incised DNA

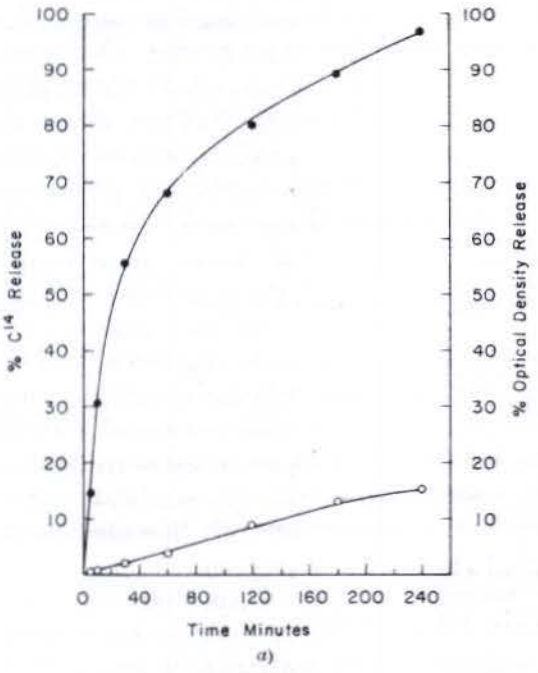
Description	Phosphomonoester groups (μμmoles) Derived from:	
	UV endonuclease	Polynucleotide-kinase
UV'd DNA + UV Endonuclease	234	—
Photoreactivated UV'd DNA + UV Endonuclease	20	—
UV'd DNA, UV endonuclease + Kinase ± phosphomonoesterase	—	25
UV'd DNA + UV endonuclease > photoreactivation + Kinase ± phosphomonoesterase	—	206.4

The nucleotide distance removed from the damaged region relative to the site of endonucleolytic hydrolysis is currently under investigation with the use of the polynucleotide kinase and ^{32}P-ATP.

Mechanism of exonuclease action

In order to determine from which end of single stranded DNA the enzyme initiates hydrolysis two species of DNAs were synthesized. One, a 3' -terminally labeled DNA was prepared by incubating DNA, ^{3}H-dATP and the *E. coli* DNA polymerase. Under these conditions only a single nucleotide is incorporated at the growing end (3' -OH end) of each DNA strand. Thus, each separate strand of a DNA molecule bears a labeled dAMP at its 3' -OH end. Labeling the 5' -OH end of each strand of DNA was accomplished with γ^{32}-P-ATP, DNA and polynucleotide kinase. This enzyme in the presence of ATP catalyzes the phosphorylation of only 5'-OH groups of such polymers.

Incubating these specifically labeled substrates with the UV exonuclease and measuring the rate of label release as a fraction of total nucleotide release provides a measure of terminus specificity. The results of such experiments are given in Fig. 7 (*a*, *b*). In each case studied, when 75 per cent of the label was released from the 3′- or 5′- end less than 5 percent



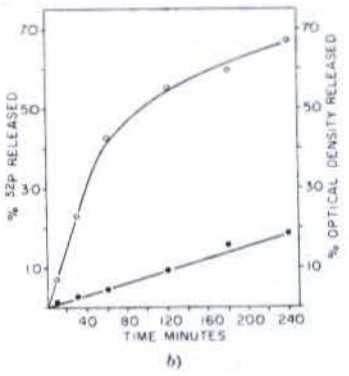


Fig. 7. — *a*) Ratio of release of 3′-terminally labelled DNA to total nucleotides.

b) Ratio of release of 5′-terminally labelled nucleotides to total nucleotides from DNA.

of the total internal nucleotides were hydrolyzed. It is concluded from these data that this exonuclease initiates hydrolysis from *both* ends of the DNA chains.

These results were unexpected in that no precedent has been provided for such a unique mechanism of exonucleolytic hydrolysis, with the exception of one case. The DNA polymerase from *E. coli* in addition to its polymerizing properties has two exonuclease activities associated with this highly purfied protein. There is the $3' \rightarrow 5'$ exonuclease activity which acts on native DNA whose activity has been ascribed to a hydrolytic reversal of the polymerizing property of the enzyme (KORNBERG, 1969). In addition there is a $5' \rightarrow 3'$ exonuclease activity for single stranded DNA and such an activity is presumed to be located at a different catalytic site on the protein from the two former activities. Apparently the $5' \rightarrow 3'$ exonuclease can also in the course of its normal hydrolytic functions remove $\widehat{TT}$ dimers in its path (KELLY *et al.*, 1969).

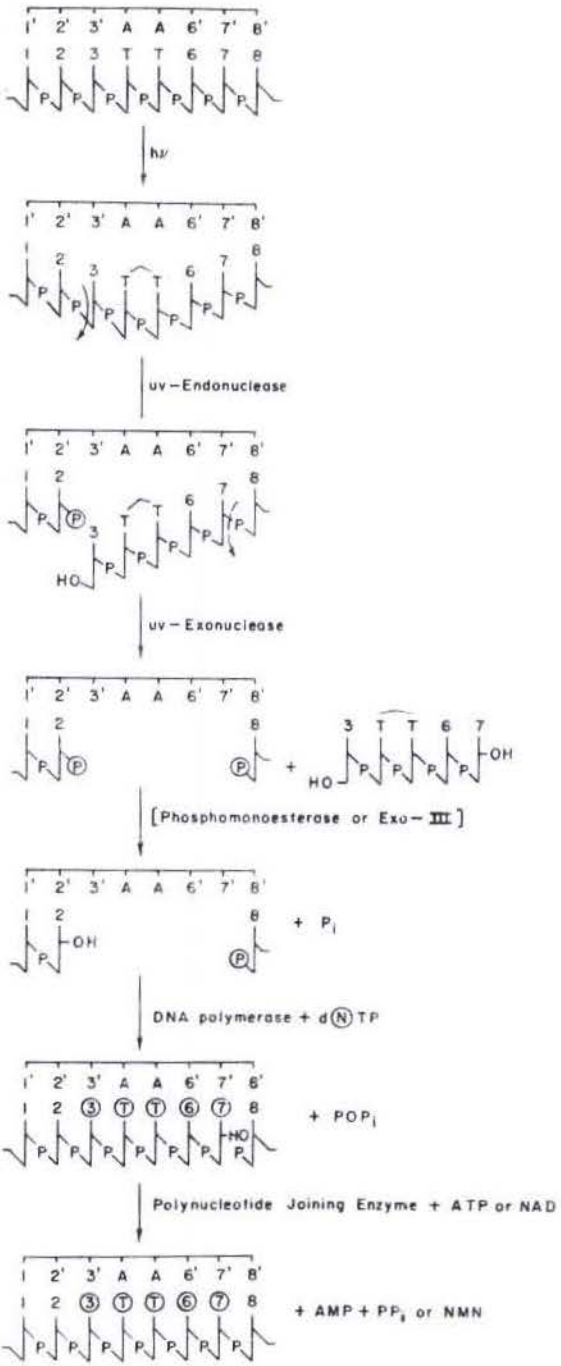


Fig. 8. — Scheme for total repair of UV damaged DNA.

It seems likely, therefore, that the UV exonuclease of *M. luteus* an excision enzyme, may be the equivalent of a subunit of the polymerizing machinery or system from *E. coli*. The UV exonuclease from *M. luteus* is devoid of any detectable polymerase activity. Morever, a DNA polymerase has been isolated from *M. luteus* which is free of any nuclease activity.

At this juncture two early enzymatic steps are elucidated in the repair chain and precedent would permit a heuristic scheme for the enzymatic repair of UV damaged DNA. (Fig. 8).

From stereochemical and physiological considerations it is most likely that UV causes distortion in the strand containing thymine photoproducts. An initial incision step is catalyzed by a specific UV endonuclease breaking this strand 5′- to the damage leaving a 3′-phosphoryl group on the DNA and a 5′- hydroxyl group at the damaged site. The resulting single stranded branch becomes susceptible to the action of the UV exonuclease liberating a small fragment which

may be further degraded to nucleotides and a yet smaller fragment . . . The total number of nucleotides released approximates five per initial nucleotide break.

The resulting gap in the DNA is terminated by phosphomonoester groups at its 3′ and 5′ termini. Polymerization by the DNA polymerase most likely synthesizes 5′ → 3′ requiring preliminary removal of the 3′-phosphoryl group for polymerization to occur. The gap is then probably filled by a DNA polymerase in which new nucleotides are inserted to the end of the space left during the excision process. Final phosphodiester bond formation is completed by a polynucleotide joining enzyme, resulting in completely repaired DNA.

Many of these enzymes are currently under study as well as their importance with respect to control loci.

Involvement of nucleases in repair in vivo

Even though the individual and combined activities of the two enzymes already discussed in this paper are consistent with the current models for the removal of damaged regions in UV irradiated DNA from *in vivo* studies, it must be established that they do, in fact, participate in cellular repair processes. Establishment of such a correlation was provided from an examination of the properties of a series of mutants and transformants of *M. luteus*.

A comparative study was performed with three strains of *M. luteus* including the wild type (ATCC-4698), a mutant obtained from this strain with N-methyl-N′-nitro-N-nitrosoguanidine (NSG) identified as ML-7$^-$ and a transformant ML-7$^+$ obtained by transforming ML-7$^-$ with wild type DNA (MAHLER & GROSSMAN, 1968).

Each strain was examined with respect to its sensitivy to UV and X-rays, the ability of such cells to repair irradiated bacteriophage B_4 (host cell reactivation, HCR), the fate of thymine-thymine dimers in its DNA during postirradiation growth periods, the resulting single strand interruptions arising from the excision process, the presence of interruptions in newly synthesized DNA (F_1) and the inhibition of DNA synthesis. These physiological properties were examined to determine whether they in fact reflect the influence of the UV endo- and exonucleases.

Table 8 presents, in summary form, the results of a large number of experiments involving these three strains. There is a direct correlation between the resistence to UV radiation and the level of UV endonuclease in these organisms. Initial experiments of UV endonuclease were made on crude extracts of these organisms with the result that the level in ML-7$^+$ was marginal and equivocally negative. Purification of the UV endonucleases

was followed to and including phosphocellulose chromatography (Fraction 3. Table 1), revealing significant, but lowered levels of the enzyme in ML-7$^+$ which had 10 percent of the specific activity of the parental wild type strain. Similar purification procedures of ML-7$^-$ did not yield any detectable levels of the enzyme. That ML-7$^+$ in a single genetic transformation step produced only a portion of the enzyme activity is currently interpreted in terms of a multiple genetic system which may be expressed in terms of a multiple protein system.

TABLE 8.

Properties of « Micrococcus luteus » strains

Strains	Radiation sensitivity			UV endonuclease S.A. (*a*)	UV exonuclease	TT excision removed (*b*)	Replication Delay Time (ergs/mm^2) (*d*)		Postreplication Repair (*c*)
	UV	HCR	X-RAY				48	240	
Wild type . NSG	+	+	+	8076.9	+	50	0′	23″	+
ML-7− . . . TRS.	—	—	—	Non-detectable	+	< 1	60′	> 240	+
ML-7+ . . .	+	+	+	854	+	20-25	15′	50	+

(*a*) Specific activity of the UV endonuclease partially purified to and including phosphocellulose chromatography.

(*b*) The percentage of thymine dimers remaining in DNA ; measured chromatographically.

(*c*) Measurement of sedimentation properties of post-irradiated labelled cells in alkaline sucrose gradients.

(*d*) Measurements of ^{3}H-TdR uptake into an acid-soluble form.

The possible involvement of the UV exonuclease enzyme in the replicating system is currently supported by the enzyme's presence in 50 mutants examined to date. It is apparent that the variability in radiation sensivitivity is not directly controlled at this enzymatic locus. That the final physiological expression of the UV endonuclease leads to radiation responsiveness is also reflected in the extent to which thymine photoproducts are removed from the cell's DNA. It is rather interesting that in the UV resistant wild type which contains the most ideal repair conditions only 50 percent of the thymine-thymine dimers are in fact removed. That there is no excess DNA in this organism certainly points to other contributing mechanisms of repair in this organism. The total number of dimers excised is a reflection of the level of endonuclease present. Similarly, the mechanisms accounting for repair synthesis of the excised regions reflect the endogenous

levels of UV endonuclease. Normally there is a very rapid onset of ^{3}H-thymidine incorporation into the new DNA with only slight delay for the initiation of repair synthesis. The delay in such incorporation is, again, a reflection of endonuclease levels. As expected there is a complete block to such synthesis when the enzyme is absent thereby implicating the necessity of initial breaks for such repair synthesis.

An examination of the hydrodynamic properties of newly synthesized DNA reveals the presence in these strains of post-replicative repair activity (RUPP & HOWARD-FLANDERS, 1968). Although theoretical considerations at this point would allow both the excision and postreplicative repair activities to fully restore viable DNA it seems clear that these systems are in fact interdependent such that each is necessary for the survival of the cell when its DNA is damaged. It follows then that a search for mutants which, though defective in the postreplicative repair machinery, contain the normal complement of excising enzymes, is indeed desirable.

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