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THE FATE OF YEAST MITOCHONDRIAL DNA AFTER ULTRAVIOLET IRRADIATION

I. DEGRADATION DURING POST-UV DARK LIQUID HOLDING IN NON-NUTRIENT MEDIUM

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SUMMARY: A partial recovery of ultraviolet (U.V., 254 nm) induced petite mutation (ρ^-) is observed in exponential phase yeast. This process requires a period of dark holding (LH) in non-nutrient medium followed by growth in nutrient medium. At intervals during LH prelabelled DNA was examined by equilibrium cesium chloride gradients. The gradual decrease in ρ^- was accompanied by an ongoing degradation of mitochondrial DNA (mitDNA) during the first 24 hours followed by a stabilization. The dose response for mitDNA degradation was biphasic. No new synthesis of mitDNA occured during LH. MitDNA remaining after degradation showed a) slight shift to a heavier buoyant density indicating a possible selective degradation of A-T regions b) no difference in size when compared to non-irradiated samples. The first step in the recovery of the ρ^- mutation is mitDNA degradation followed by other events taking place when growth resumes.

A controlled excision of UV-induced pryimidine dimers does not operate on mitDNA of irradiated yeast cells at high (1) or low (2) doses. The same is true for mammalian cells mitDNA (3). A loss of mitDNA occurs in UV-irradiated stationary phase yeast after dark liquid holding (LH) (1). This is accompanied by an enhancement in the frequency of ρ^- mutants (4). However the fate of the respiratory genetic determinant equated to mitDNA is not irrevocably determined after a UV treatment since a) nuclear or mitochondrial mutants UV sensitive to ρ^- induction have been identified suggesting that wild type cells are able to reverse a fraction of the UV induced mitochondrial damage (5, 6) and b) the LH of UV treated exponential phase cells is followed by a diminution in the frequency of ρ^- mutants (4). Moreover inhibitors of nuclear and mitochondrial directed protein synthesis or inhibitors of repair were shown to interfere with the LH response for ρ^- UV induction (7, 8). The possibility therefore of an accurate repair mechanism of UV damaged mitDNA for exponential phase cells is still open and even the degradation of mitDNA in UV treated stationary phase cells could be

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Abbreviations: mitDNA, mitochondrial DNA; UV, ultraviolet; ρ^-, respiratory deficient "petite" mutant; LH, liquid holding; CH, cycloheximide; YEP, 1% yeast extract, 2% Bacto-peptone, 2% glucose.

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viewed as an unsuccessful attempt at repair. We performed a closer examination of the behavior of mitDNA in UV treated exponential phase cells during the LH period.

MATERIALS AND METHODS: Strain, Radioactive labelling, UV irradiation and Liquid Holding.

The haploid strain of Saccharomyces cerevisiae N 123 (a hi_1) was used. It has a wild type response to UV and a spontaneous frequency of ρ^- mutants from 1-3%. Aerated cultures were grown to exponential phase (2-3 x 10^7 cells/ml) at 30°C in Difco minimal medium of yeast nitrogen base, 2% glucose and 40 µg/ml histidine. For preferential radioactive labeling of mitDNA 1 µg/ml CH was added to the growing culture to arrest protein synthesis and slow down nuclear DNA replication (9) without interfering with chase of the radioactive label and normal recovery after UV. After a 75 min incubation in the presence of CH, 8 µCi/ml of [6-^{3}H] uracil (spec. act. 26 Ci/mM) were added for a 2 hr labeling. The average increase in cells during this 3 hr 15 min period was 20%. The cells were then collected by centrifugation, washed (3x) in tridistilled water containing 50 µg/ml cold uracil (chase mixture) and aerated for 30 min at 30°C. Following a wash by centrifugation in water containing 10 µg/ml cold uracil, the cells were sonicated 30 sec to disrupt clumps and suspended in the same solution at 5 x 10^6 cells/ml for irradiation and LH. Cell suspensions of 50 ml were irradiated with stirring in 200 x 30 mm Petri dishes at 254 nm with an incident dose of 25 ergs/mm^2/sec as measured by a Latarjet dosimeter. Irradiated and non-irradiated samples were kept in the dark with shaking at 30°C up to 40 hrs. Immediately after irradiation and at increasing time intervals during LH aliquots of 10^8 cells were withdrawn, centrifuged, resuspended in 6 ml of "crushing mixture" (see below) and frozen in an ethanol dry ice mixture. In some experiments exact volumes of irradiated cells were added to separate flasks containing premeasured amounts of drugs to investigate the effect of drugs on LH recovery and samples were treated as described above. Cellular responses immediately after UV irradiation and during LH were determined by plating appropriate dilutions of cells onto YEP agar plates. After a five day growth at 30°C colonies were overlaid with tetrazolium (10) and scored for grandes, sectores and ρ^- colonies. A mean of 600 colonies per point were counted.

DNA Preparation, equilibrium and velocity gradient sedimentations

The isolation of DNA was done according to the procedure of Williamson *et al.* (11) with slight modifications. Cells were frozen for storage in a "crushing mixture" of 10% (v/v) glycerol containing 0.01 M $MgSO_4$, 3 mg/ml sulphonated polystyrene (Polysciences Inc., Pa.) and 0.05 M succinate buffer, pH 5.0. To prepare DNA each cell suspension (10^8 cells) was thawed, transferred to a cold Eaton press and crushed. The crushed material was thawed and centrifuged 40 min at 15,000 x g at 4°C. The pellet was lysed at 60°C for 10 min by addition of 2 ml of 0.25 M Tris-HCl buffer (pH 8.0) with 0.1 M EDTA and 1% Sarcosyl. The mixture was centrifuged 40 min at 15,000 x g and the supernatant containing DNA, RNA and protein was extracted once with chloroform: isoamyl alcohol (24:1) and centrifuged 20 min at 12,000 x g at 4°C. The aqueous layer was removed with a large bore pipette. RNA was digested by the addition of 0.1 ml bovine pancreas RNAse (Sigma, 1 mg/ml) and T_1 (Sigma, 500 units) with 1 hr incubation at 37°C. The DNA solution was dialyzed two days with numerous changes in SSC (0.15 M NaCl and 0.015 M sodium citrate) with 10^{-3} M EDTA, pH 7.0 in the cold. Cesium chloride solutions for equilibrium centrifugation were prepared by the addition of each sample (6 ml) to a polyallomer tube containing 7.210 g CsCl. Tubes were centrifuged 60 hrs at 42,000 rpm in a fixed angle 50 rotor in a Beckman L-4 ultracentrifuge at 20°C. Fractions were collected with an ISCO gradient fractionator by pushing the gradient from the bottom with fluorinert. Samples were collected in scintillation vials and counted for radioactivity after the addition of 0.5 ml water and 9 ml of NE 260 toluene base liquid scintillator (Nuclear Enterprises Ltd). For samples used for velocity sedimentation fractions were collected in glass tubes and the radioactivity present in 0.01 ml from each tube was counted to determine the fractions containing mitDNA. These fractions were pooled and dialyzed overnight in SSC with EDTA to remove CsCl. MitDNA from dialyzed fractions (2-3 ml) was concentrated to 0.1 ml. Linear sucrose gradients (4.9 ml) of 5% to 20% (w/w) in 0.8 N NaCl, 0.2 N NaOH were prepared in polyallomer tubes. A top layer of 0.1 ml NaOH was applied to the gradient followed by 0.1 ml concentrated DNA solution. After 20 min the gradients were spun to 20,000 rpm for 18 hrs at 20°C in an SW 50 rotor. The fractions were collected into scintillation vials. To neutralize the NaOH 0.5 ml of 5 N HCL was added to each vial followed by 9 ml of scintillation fluid.

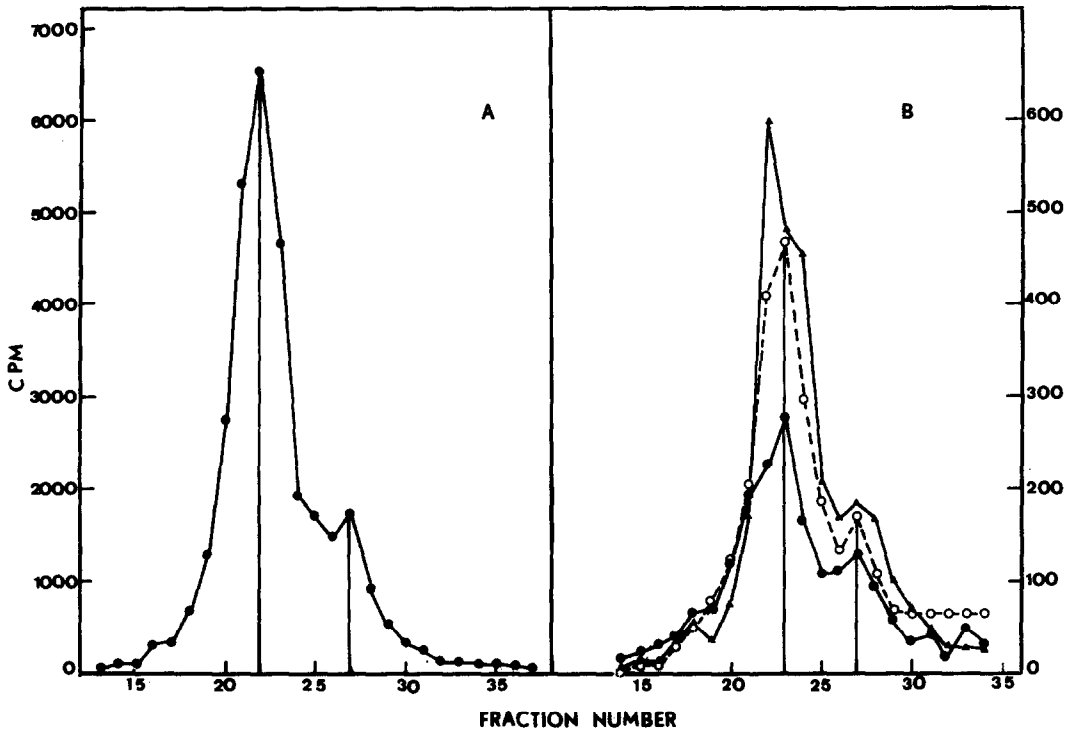

Fig. 1 : Separation of yeast nuclear (fractions 25 to 30) and mitochondrial DNA (fractions 18 to 24) by CsCl density gradient centrifugation from non-irradiated cells (A) and UV-treated cells (B). The incident doses were ▲ 200 ergs/mm^2, o 500 ergs/mm^2, ● 1000 ergs/mm^2. Cells were labeled with [6-^{3}H] uracil in the presence of cycloheximide. Total DNA was extracted after a 40 hour post-irradiation dark liquid holding period in water.

RESULTS: Figure 1A shows the distribution of radioactivity between mitochondrial and nuclear DNA in equilibrium CsCl gradients from one representative experiment. To determine the percentage of degradation of mitDNA after different UV doses and 40 hours of LH the ratio of radioactivity between mitochondrial and nuclear DNA was calculated (M/N). In non-irradiated cells this ratio which remained constant during LH varied from values of 2 to 4 in each individual experiment. In order for these ratios to reflect reliable fluctuations in mitDNA, the nuclear DNA radioactivity was considered as a constant value with minimal changes during LH. This was confirmed by the addition of 10^8 cells in exponential phase labelled for two generations without CH in [^{14}C] adenine to samples of ^{3}H-labelled cells (as described above) both irradiated and non-irradiated. DNA from these cells was separated on CsCl gradients, the ^{14}C-DNA

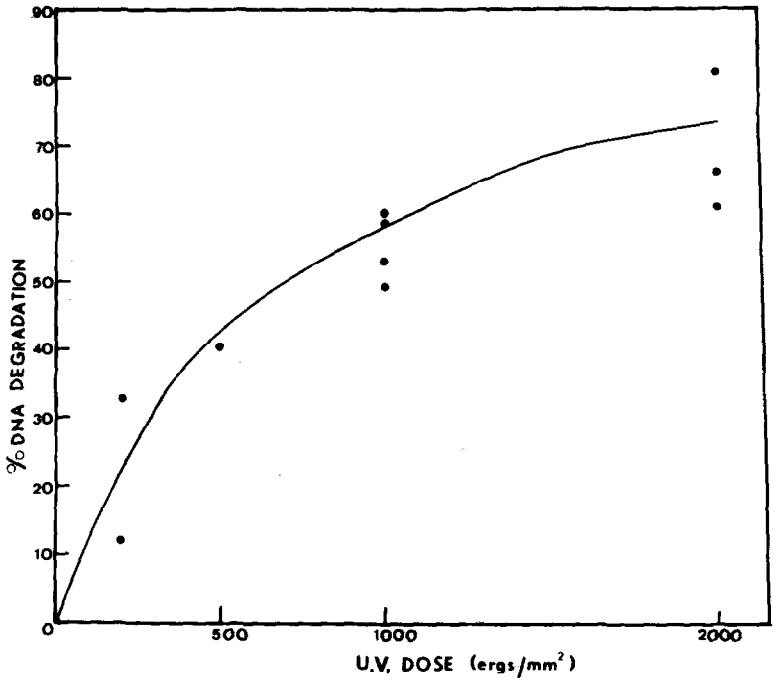

Fig. 2 : Final percentage of mitochondrial DNA degradation as a function of UV-dose after a 40 hour liquid holding period. Multiple points at different doses represent the results of several independant determinations.

serving as an internal control to examine possible changes in nuclear DNA radioactivity. The counts of ^{3}H were calculated for each gradient by standardizing them to the amount of ^{14}C present. The percentages of degradation using the M/N ratios or the ^{3}H counts determined for each component by the internal control of ^{14}C showed good agreement within a 10% fluctuation. This ratio approach is further justified by the demonstrated stability of nuclear yeast DNA during LH following UV irradiation, e.g. at doses up to 4000 erg/mm^2 no more than 7 to 10% of the total DNA was degraded (12). Hence the reduction in the M/N ratio was judged to be a reliable monitor of mitDNA degradation during LH. The amount of mitDNA remaining after 40 hours of LH decreases with increasing doses of UV (fig. 1B). Figure 2 indicates the dose dependance of degradation. The curve depicts a two component response for degradation with different slopes. Similarly, as often shown, a biphasic curve for ρ^- UV induction is seen in immediate and delayed plating (fig. 3). The preincubation of cells with 1 µg/ml of CH does not modify the

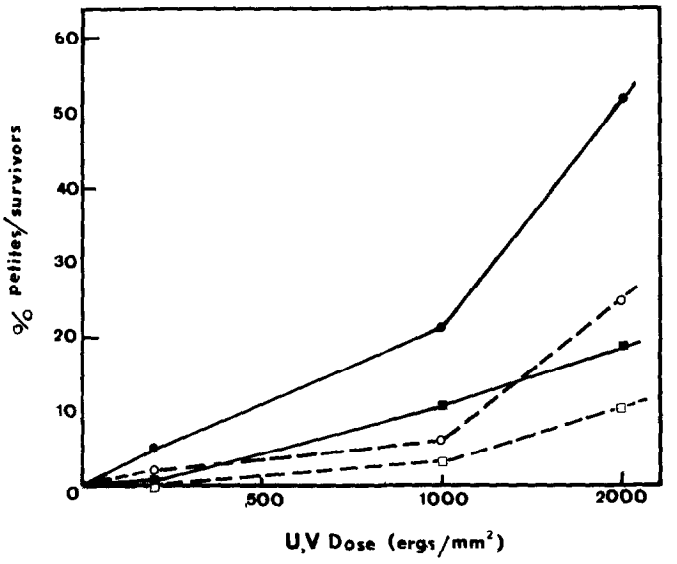

Fig. 3 : Percentage of respiratory deficient mutants (ρ^-) among the survivors as a function of UV-dose on immediate plating (●, o) or on delayed plating (■, □) after 40 hours of liquid holding in water. Full symbols correspond to sectored plus complete ρ^- colonies (——) whereas empty symbols correspond to only complete ρ^- colonies (---).

Table 1. Changes in the UV induced "petite" population during LH recovery in exponential phase yeast. P + S = % "petites" and sectored colonies ; P = "petites" only ; A and B represent the results from two separate experiments.

Time of LH (hours)	Dose - 1000 ergs				Dose - 2000 ergs			
	P + S		P		P + S		P	
	A	B	A	B	A	B	A	B
0	19	20	4	5	39	52	14	25
17	25	21	4	6	38	--	15	--
20	18	--	3	-	32	--	14	--
24	18	17	3	5	31	--	13	--
40	13	11	2	4	28	19	14	10

dose response for ρ^- induction as compared to untreated cells (data not shown).

Figure 4 shows the time course of mitDNA degradation during LH after a dose of 1000 er

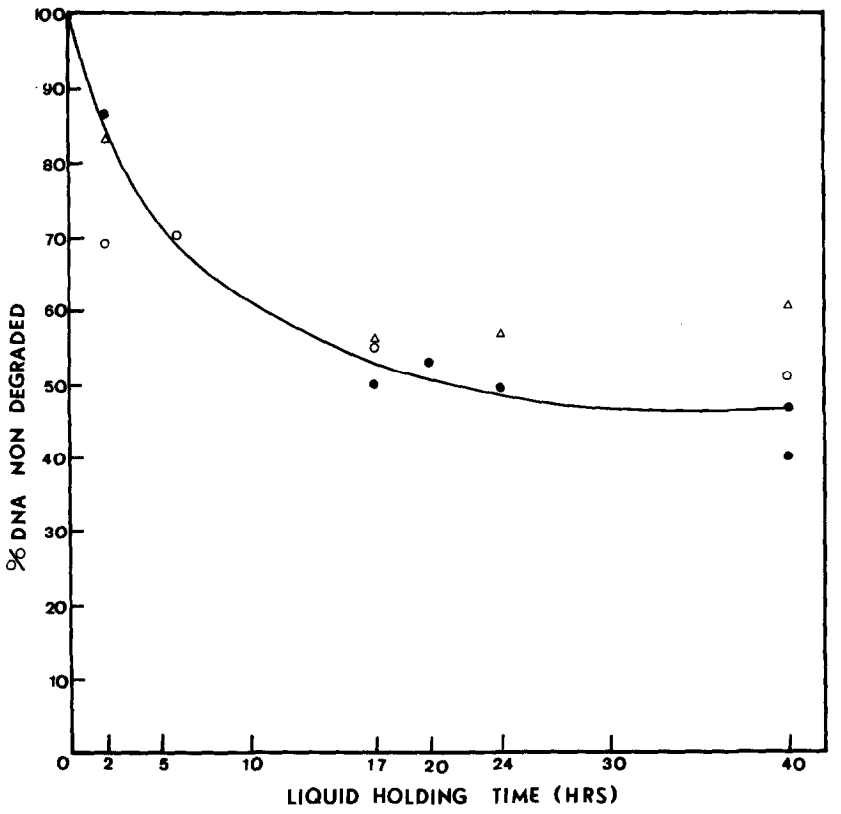

Fig. 4 : Percentage of mitochondrial DNA non-degraded as a function of time of dark liquid holding after a constant dose of UV of 1000 ergs/mm^2. The symbols (●, Δ, o) correspond to sets of determinations done on three different cell batches.

mm^2. A gradual increase in degradation is shown which reaches a plateau at 40 hour LH. A corresponding decrease in ρ^- mutants is seen (Table 1). Analytical centrifugation of DNA after LH showed a close agreement between the amount of DNA degraded after LH and the amount of mitDNA as measured by densitometer tracings. Consequently the degradative process occuring for mitDNA in non-growth medium is not accompanied by detectable new synthesis. In irradiated samples as compared to non-irradiated the position of the mitDNA peak with respect to the nuclear peak in equilibrium CsCl gradients is skewed in the heavy density direction (fig. 1 and 5). Since this is observed in all experiments, it may indicate that the A-T rich segments are more subject to degradation than the rest of the mitochondrial genome. The role of nuclear or mitochondrial directed protein synthesis on mitDNA degradation after UV was studied by the addition of CH (1 µg/ml) or

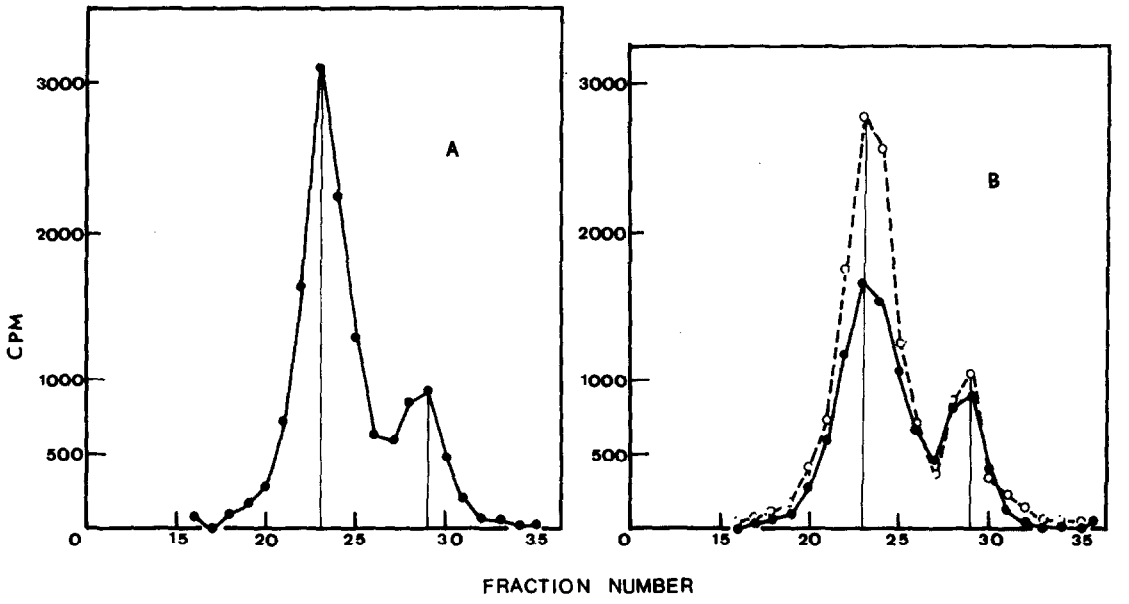

Fig. 5 : CsCl density gradient centrifugation of non-irradiated cells (A) and cells treated with a dose of 1000 ergs/mm^2 (B) after 2 hours (o) and 40 hours (●) of dark liquid holding.

chloramphenicol (4 mg/ml) to the LH medium. The LH recovery of cell survival was inhibited by both drugs and the ρ^- induction response was also modified (7,8). Chloramphenicol appeared to inhibit the degradation of mitDNA during LH whereas CH demonstrated either no effect or a slight inhibition (table 2). Alkaline sucrose gradients were used to examine the mitDNA after UV and LH. As reported by other workers the mitDNA from non-irradiated cells was nicked in spite of the precautions taken. No differences can be seen between the molecular weights of mitDNA of irradiated cells and unirradiated cells (fig. 6); this is true for different doses and lengths of time of LH.

DISCUSSION: The liquid holding recovery after UV irradiation involves two steps; the first takes place in non-nutrient medium and the second in growth medium. The response for ρ^- induction in exponential phase cells is a recovery of the wild type phenotype. For such irradiated cells the fate of mitDNA in non-nutrient medium was examined. The major event is a degradation of this DNA which appears to favor A-T rich segments. The pattern of degradation is biphasic in terms of dose dependance and timing of LH. In the low dose range the degradation mounts quickly in relation to dose while at higher

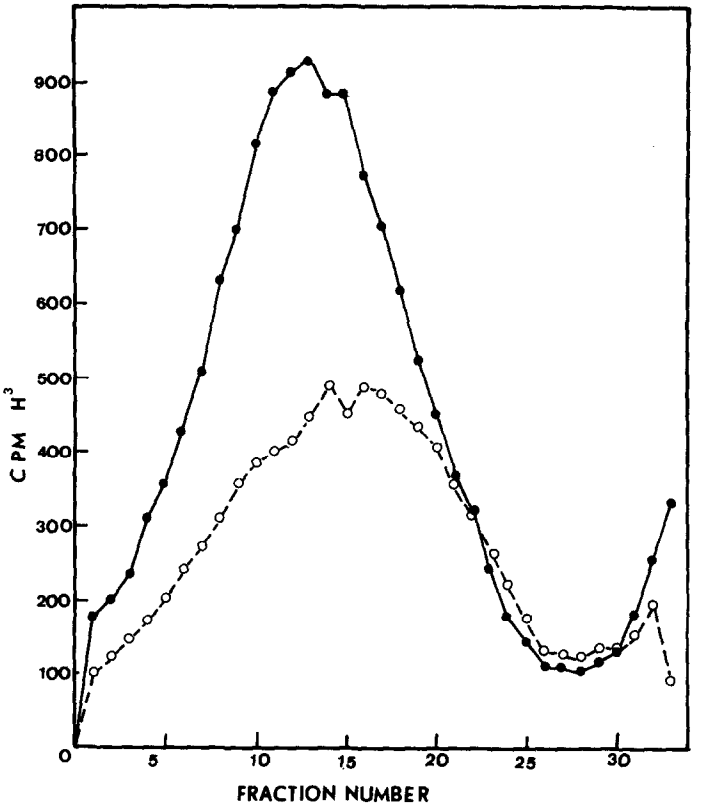

Fig. 6 : Sedimentation of mitochondrial DNA in a sucrose gradient from non-irradiated cells (-●-) and cells treated with 1000 ergs/mm^2 (..o..). A period of 40 hours of liquid holding in water is given to the cells before the DNA extraction. Sedimentation is from left to right.

Table 2: Drug effects on UV irradiated cells after 1000 ergs/mm^2 and 40 hours of liquid holding (two independent experiments). cycloheximide (CH) 1 µg/ml; chloramphenicol (CAP) 4 mg/ml; P + S, percentage of "petite" and sectored colonies after 40 hours of LH.

	% P + S/survivors	% mit DNA degradation
1000 ergs/mm^2	40	none
1000 ergs/mm^2 + 40 hrs. LH	24	49
	11	60
1000 ergs/mm^2 + 40 hrs. LH with CH	29	51
	19	30
1000 ergs/mm^2 + 40 hrs. LH with CAP	43	none
	42	26

doses it is not as responsive. The dual response seen for ρ^- induction is likely to be related to the differences in degradation at high and low doses. The biphasic curves suggest a saturation of the enzymatic reaction(s). The resistant component cannot be due to the absence of UV-induced lesions in a fraction of the mitDNA population since for a dose of 1000 ergs/mm^2 60 dimers are present per mitDNA molecule (1). Such a saturation effect is documented for other enzymatic repair processes (13). From the analytical centrifugation data a concomittant new synthesis of mitDNA does not seem to occur. Inhibitors of protein synthesis inhibited mitDNA degradation after UV, chloramphenicol being more effective than CH. However the concentration of CH used probably allowed a low level of protein synthesis. For chloramphenicol a large inhibition of mitDNA degradation was shown. The modulation in ρ^- induction after LH in the presence of the drugs may be due to a selection among the survivors after UV. However both cytoplasmic and mitochondrial protein synthesis are necessary for the degradation of mitDNA after UV. The nature of the retained mitDNA after LH may be questioned. Either in each of the mitDNA molecules a portion is degraded or a part of the pool of mitDNA molecules is intact while another part is degraded. Information on the characteristics of the retained mitDNA molecules is essential for an understanding of the processes taking place in the growth medium which complete the steps of LH recovery.

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