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The Effect of Colloidal n-C_{60} on DNA Mutagenesis and the SOS DNA Repair Mechanism of *Salmonella typhimurium* strains TA1538 and TA1535

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The Ames test was utilized to examine the effect of n-C_{60} on growth, mutagenicity, and DNA repair mechanisms. Nano-C_{60} was prepared using the THF solubilization method and tested against Gram-negative histidine mutant *Salmonella typhimurium* strains TA1538 and TA 1535, at concentrations of 0.04, 0.12, 0.21, 0.26, 0.43, 0.53 mg/L to test for effects on growth and mutagenicity. UV treatment at 118 Jm^{-2} was used to induce the SOS DNA repair response to investigate the effect of n-C_{60} on DNA repair mechanisms. No apparent growth inhibition was observed with both strains grown under varying concentrations of n-C_{60} and no mutagenicity was seen with TA1535, but the classification of n-C_{60} as a mutagen was inconclusive with TA1538 grown under the same conditions. Lack of growth on UV treated plates is likely due to a lethal UV dosage and therefore the effect of n-C_{60} on the SOS DNA repair response was not determined.

Over the last decade, much interest has been garnered into the fullerene family of compounds for their unique chemical and physical properties. Structurally fullerenes are characterized by a hydrophobic carbon sphere composed of 60 or 70 carbon atoms that form stable carbon-skeleton structures (14). These characteristics may be useful in a number of biological applications, from drug delivery to antiviral therapy as a protease inhibitor in the treatment of HIV (2, 3, 7, 12). C_{60} (powder form) is the best characterized fullerene but is found to have extremely low solubility ($<10^{-9}$ mg/L) in polar solvents such as water (3). However, upon extended exposure to such solvents, it forms a "water-stable, colloidal aggregate" with higher solubility (100 mg/L), referred to as nano-C_{60} or n-C_{60} (3). The increased solubility of n-C_{60} makes it the more biologically relevant form.

Increased levels of production have led to environmental and health concerns. As the potential applications of fullerenes in nanotechnology are immense, research on these applications have far preceded conventional toxicology and mutagenicity studies. In fact, data on the long-term effects of fullerenes is completely lacking (12). A handful of recent studies however have been done which highlight the potential hazardous effects of n-C_{60} and n-C_{60} derivatives. Both n-C_{60} and its polyhydroxylated derivative have been shown to be toxic in mouse and human cell lines, with n-C_{60} inducing rapid ROS-associated necrosis characterized by cell membrane damage (11). Autophagosome associated cell death has been demonstrated in human cell lines exposed to n-C_{60}(OH) (13). Furthermore, *in-vivo* studies exposing large mouth bass to n-C_{60} induced peroxidation of neural membranes (10). While the studies have attributed cytotoxicity to membrane damage, few studies have investigated the potential affect of C_{60} fullerenes on DNA.

Computer simulations have predicted the binding of C_{60} to the exposed ends and minor groove sites of doubled stranded DNA and an even more pronounced effect on single stranded DNA (14). Together, these observations suggest potential adverse affects on DNA processes, including DNA synthesis and repair. Thus, C_{60} may have important growth inhibitory and mutagenic properties that warrant further investigation.

The Ames test was performed to assess the mutagenic and indirectly the carcinogenic potential of n-C_{60}. The Ames short-term test (STT) is the predominant biological assay for detecting and assessing the genotoxic and mutagenic effects of a process by measuring the number of prototroph revertants. *Salmonella typhimurium* strains contain auxotrophic *his* mutations which can be reverted if the compound tested is mutagenic. In addition to investigating the mutagencity of n-C_{60}, its potential inhibitory role on DNA repair mechanisms was investigated by inducing the SOS response in TA1535 and TA1538 strains of *S. typhimurium* through UV irradiation.

MATERIALS AND METHODS

Strains. The supplied strains were labeled as *S. typhimurium* strains LT2 (wild-type), TA98 (*hisD3052*, Δ*uvrB, rfa,* pKM101*)* and TA100 (*hisG46*, Δ*uvrB, rfa,* pKM101*)*. Phenotypic characterization for *his* mutation, *uvrB* deletion, *rfa* mutation, and pKM101 plasmid were determined using standard growth tests (4) which indicated that the auxotrophs required histidine

but were not ampicillin resistant. The strains TA1538 and TA1535 are identical to strains TA98 and TA100 respectively except that they lack the plasmid pKM101 and are thus sensitive to ampicillin (4). Therefore, the auxotrophic strains will be referred to as TA1538 and TA1535 throughout the report.

Preparation of *S. typhimurium* strains. This procedure was modified from Mortelmans *et al.* (9). The TA1538 and TA1535 strains were provided by the MICB 421 teaching lab frozen stock cultures from the University of British Colombia. Exposure of the TA1538 and TA1535 strains to light was minimised at all times. The following isolation and phenotypic confirmation was performed on each of the three strains (LT2, TA1538 and TA1535) unless otherwise specified. The following sub-plating procedures were performed on only the TA1538 and TA1535 strains. An isolated colony was picked and plated on minimal agar supplemented with an excess of biotin and histidine (MABH) as previously described (9). It was then incubated at 37ºC for 48 hrs. This purification was done three times to ensure that a pure culture was isolated. Overnight cultures of all three strains for the phenotypic characterization were prepared by inoculating a test tube containing 5 ml of Luria-Bertani, LB, broth (2 g tryptone, 1 g yeast extract, 1 g NaCl, 0.6 g glucose, 200 ml dH_2O, pH 7.0) with one isolate from each of the 5 sections on the 3rd MABH plates and with an isolate from the LT2 MABH plate. Cultures were incubated for ~15 hrs at 37ºC and ~120 RPM. Cultures were then pelleted and resuspended in phosphate-buffered saline, PBS, (0.2 M $NaH_2PO_4 \cdot H_2O$, 0.2 M Na_2HPO_4, pH 7.4) to avoid erroneous phenotypic results caused by the LB broth as previously described (9). Phenotypic characterisation was performed to confirm the presence of the *his* mutation, *uvrB* deletion, *rfa* mutation, and the pKM101 plasmid in TA1535 and TA1538 as previously described (9). To test for the presence of the *uvrB* deletion, biotin dependence was tested instead of sensitivity to UV as the deletion in the *uvrB* gene is a large deletion of 50 kb in TA1535 and 125 kb in TA1538 which extends into the *uvr, bio, chl* and *gal* operons (overlapping) (9).

Overnight for Ames Test. An isolated colony was picked from each of the 3rd MABH TA1538 and TA1535 plates, and used to inoculate 25 ml of LB broth. Cultures were incubated for ~22 hrs at 37ºC in the dark in a shaker bath set at ~120 rpm. The cultures were grown to an OD_{660} of ~0.75-0.80. Plate counts on LB were then performed in duplicate to determine the cell concentration.

Preparation of n-C_{60}. A procedure modified from Deguchi *et al.* (3) and Fortner *et al.* (7) was used. A saturated solution was produced by adding 40 mg of C_{60} (98 % pure, MW 720.64, Aldrich 483036) to 40 ml of tetrahydrofuran, THF (≥99%, Sigma T5267). This solution was then de-aerated by purging with N_2 gas at a pressure of ~500 Lbs/in^2 for 30 min and was loosely capped. The solution was left to stir overnight (~24 hrs). A turbid brown solution resulted due to excess C_{60}. Excess solid was filtered off with a glass syringe fitted with a 0.45 µm nylon membrane (Fisherbrand 09-719D), resulting in a clear uncoloured filtrate. The level of solution was then marked on the bottle and an equal volume of MilliQ water (source: Millipore MilliQ-185 from the UBC Biomedical Research Centre) was added to the bottle. A clear yellow solution formed upon addition of the MilliQ water. The bottle was immersed in a water bath set at room temperature to prevent the large temperature drop caused by the subsequent evaporation of THF. The n-C_{60} solution was purged with N_2 at a pressure of ~500 Lbs/in^2 for ≥1.5 hrs to evaporate the THF. Previous studies have shown that by using gas chromatography only <1 ppm of THF remained in an n-C_{60} following the afore mentioned adapted purging protocol (3). The inadvertent evaporation of water was compensated for by addition of MilliQ water to the previously marked original n-C_{60} solution level (3). The n-C_{60} solution now dissolved in MilliQ water was stored in the dark for the remainder of the experiment. A 5 ml aliquot of the n-C_{60} MilliQ solution was autoclaved at 121ºC for 20 min before use in the Ames mutagenicity assay.

Quantification of n-C_{60}. Nano-C_{60} was extracted from the MilliQ water into toluene for concentration determination via spectrophotometry, as previously described (3). Briefly, 0.3 ml of n-C_{60} was added to a 1.5 ml microfuge tube along with 0.3 ml of 2% NaCl solution. The solution was then vigorously vortexed once a minute for 20 sec durations for 5 min. Finally, 0.6 ml of toluene (≥99.5%) was added and the solution was vortexed as above but for 15 min. The aqueous phase was frozen by dry ice and the organic top phase was extracted and transferred to another microfuge tube. To collect the bottom organic phase, the test tube containing the aqueous and bottom organic phase was allowed to warm to room temperature; as a result the bottom organic phase rose to the top thus becoming the new top organic phase. The tube was again subjected to dry ice to freeze the aqueous bottom layer. As a result the top layer was collected and pooled with the previously collected organic phase. The absorption spectrum of the combined organic fraction was then determined using a spectrophotometer scanning between the wavelengths 240-460 nm. Beer's Law and the reported absorption coefficient ($\varepsilon=10^{4.71}$) was used to convert the observed absorption to a concentration (3).

Ames mutagenicity assay. Five different concentrations of n-C_{60} were tested in the Ames mutagenicity assay, 0.53, 0.4, 0.26, 0.2, 0.12 and 0.04 mg/L. A modified protocol from Mortelmans *et al*. (9)

TABLE 1. Mean number of revertant cells in the Ames strains after treatments

Chemical/Agent	Dosage	*S. typhimurium* Tester Strain			
		TA1538		TA1535	
		Mean	S.D.[a]	Mean	S.D.[a]
MilliQ water	N/A	11	5	18	11
UV	118 Jm^{-2}	4	5	7	12
THF	N/A	18	13	12	10
n-C_{60}	0.53 mg/L	20	4	16	6
	0.43 mg/L	12	10	15	5
	0.26 mg/L	26	10	11	0
	0.21 mg/L	9	3	17	3
	0.12 mg/L	10	2	12	3
	0.04 mg/L	4	4	17	3
n-C_{60} and UV	0.53 mg/L	1	1	2	2
	0.43 mg/L	12	12	3	2
	0.26 mg/L	1	1	1	1
	0.21 mg/L	9	2	N/A	N/A
	0.12 mg/L	1	1	0	0
	0.04 mg/L	6	2	10	6

[a]S.D., standard deviation.

was used. Due to time and budget constraints, S9 was not used. All plates were done in triplicate to allow for subsequent statistical analysis of data. Positive (UV irradiation), negative (MilliQ water) and solvent (THF) controls were used and the *S. typhimurium* tester strains were subjected to two different experimental treatments of n-C_{60}. Cells were either exposed to n-C_{60} only or to concomitant n-C_{60} and UV treatment. A standard plate incorporation method was used. In brief, aseptically, 0.1 ml of test (n-C_{60}), control (MilliQ or THF) or MilliQ (used for volume compensation in UV control samples) solution and subsequently 0.1 ml of the appropriate overnight culture was added to 2 ml molten top agar (1.2 g agar, 1.2 g NaCl, 2.48 mg D-biotin, 1.92 mg L-histidine, 200 ml dH_2O). A modified 50X Vogel-Bonner minimal media (1.0 g $MgSO_4 \cdot 7H_2O$, 10 g citric acid monohydrate, 50 g K_2HPO_4 dibasic, 4.5 g NH_4Cl, 8.5 g NaH_2PO_4, 67 ml dH_2O) was used in the preparation of the bottom agar (15 g agar, 20 ml 50X VB, 10% wt/vol glucose, 900 ml dH_2O). The top agar tubes were then gently mixed via phage style mixing and poured evenly over the corresponding bottom agar plate. The time between the removal of the top agar from the water bath and pouring of the plates was minimized to prevent the premature hardening of the top agar. The top agar was allowed to solidify (~10 min) while the plates were covered with aluminum foil to control the frequency of back mutations caused by background UV radiation. The plates that required UV treatment were then irradiated using the Stratagene Stratalinker Model 2400 (15 W bulb) at a dosage of 118 Jm^{-2} to induce a reproducible mutagenic positive result (4). The plates were then inverted and incubated for 48 hrs at 37ºC. Revertant colonies were counted by eye and the background lawns were examined for potential toxicity to n-C_{60} or UV treatment.

RESULTS

Phenotypic analysis characterized the Ames strains as following; TA1538 and TA1535 did contain the *rfa* mutation and a deletion in *uvrB* and was auxotrophic for histidine but did not contain the plasmid, pKM101. The deletion of the *bio* and hence *uvrB* gene in TA1535 was not complete as fair growth (~50% of the LT2 control strain, data not presented) was still observed in the UV irradiated TA1535 cells and also good growth (~80% of the LT2 control strain, data not presented) was still observed in the MG agar plates supplemented with histidine only. Otherwise TA1538 and TA1535 conform to the phenotypes characteristic of each strain.

A 240-300 nm absorbance scan found one peak was at ~334 nm. This corresponded to a n-C_{60} concentration of 11.55 mg/L. The pristine fullerenes remained stably dissolved in MilliQ water through the experiment. There was no evidence of precipitation in our n-C_{60} stock solution kept at room temperature for 3 weeks, nor was there evidence of a precipitate in our autoclaved n-C_{60} stock solution, as well as the top agar upon plating and incubation for 96 hours at 37°C, n-C_{60}

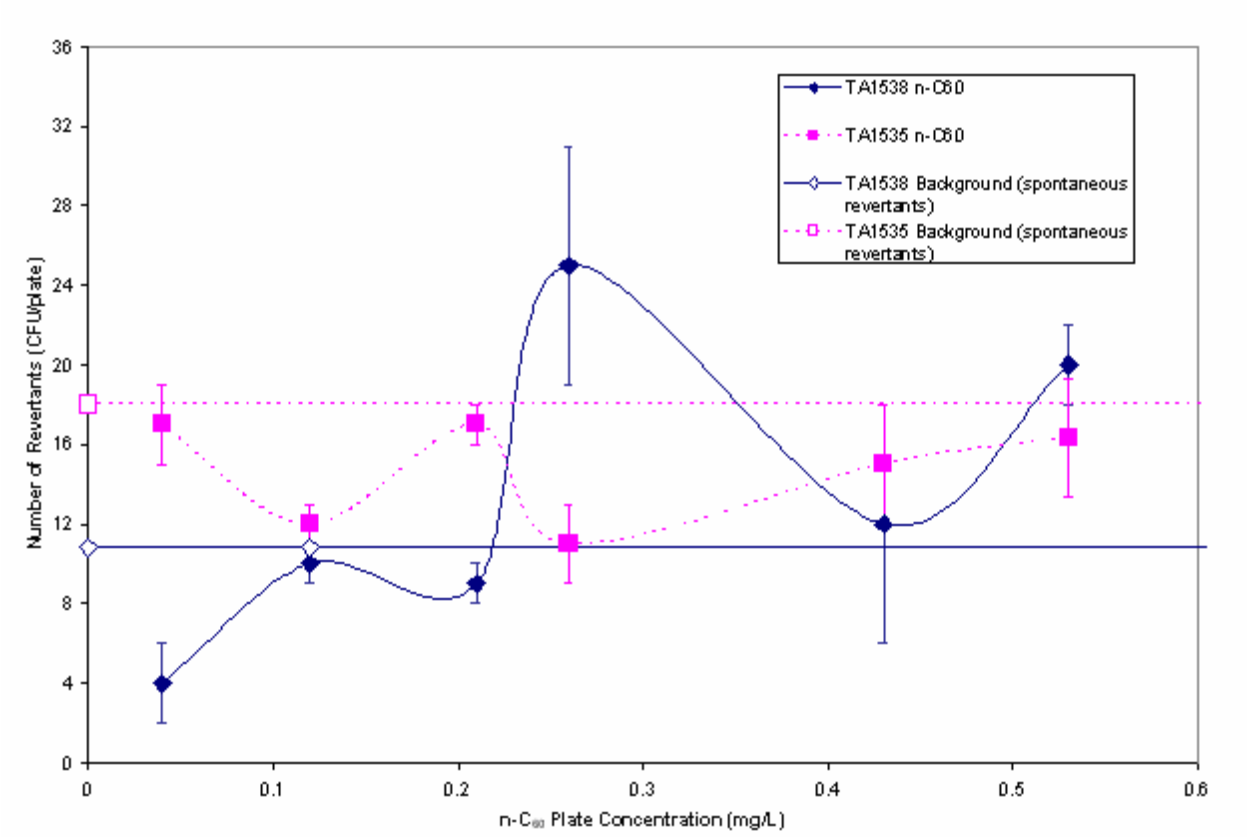

FIG. 1 Dose-response curve of Ames *S. typhimurium* strains TA1538 and TA1535 to n-C_{60} treatment. Error bars represent the standard error (S. E. M.) of each data point.

likely remained in solution, and thus biologically able to interact.

A complete absence of a background lawn was seen in the UV treated samples. It was concluded that the UV dosage of 118 Jm^{-2} which was previously used (4) to induce reversions in TA1535 was found to be consistently lethal to both TA1538 and TA1535 in this experiment (Table 1).

A thinning of the background lawn , so that individual proximal minute colonies ≤ 1 mm in diameter could be seen instead of a confluent lawn in ~20% of the total area of the plate, was observed in the THF treated samples.

The number of spontaneous revertants ranged between 15-20 for TA1535 and 3-17 for strain TA1538, respectively. In the TA1535 strain, a dose-related increase in the number of revertant colonies was not observed and the number of revertants in all n-C_{60} treated samples was lower than the MilliQ water negative control samples (Fig. 1). In the TA1538 strain, a conclusive dose-related increase in the number of revertant colonies was not seen in the n-C_{60} treated samples and only 50% (corresponding to cells that were treated at 0.26, 0.43, 0.53 mg/L) of the samples treated with n-C_{60} contained a higher revertant count as compared to the MilliQ water negative control samples (Fig. 1). The TA1538 sample treated with 0.26 mg/L of n-C_{60} was the only condition that supported a revertant count that was at least twice as high as that of the MilliQ water control.

Lines were drawn in Fig. 1 to highlight that in the TA1535 strain a dose-related response was not seen; the number of revertants showed an oscillating pattern around a mean of ~15 reverants/plate and to highlight that in the TA1538 strain a conclusive dose-related response was also not observed and instead an oscillating pattern that gently sloped upward was seen.

DISCUSSION

An initial attempt to actively induce mutation in our test strains through UV rays was unsuccessful, as the positive control group failed to grow under the 118 Jm^{-2} UV dosage. Subsequently, the data obtained from all UV treated samples was invalid and was not further analyzed. As a result the effect of n-C_{60} on DNA repair mechanisms could not be elucidated. The THF was found to be slightly toxic to the cells, as it inhibited the

growth of the background colonies on each solvent control plate. Since the THF evaporation procedure (3) that was used in this experiment was successful at removing all detectable (< 1ppm) traces of the THF the toxic effect of the THF was negligible in the in the n-C_{60} test solution. This assumption is attested as the background lawns in almost all concentrations of the n-C_{60} treated samples (0.04-1 mg/L) were confluent and were approximately as turbid as the MilliQ water control samples.

Mortelmans *et al.* proposed a historical spontaneous revertant frequency of 5-20 colony forming units (CFU)/ $2x10^8$ cells plated for both TA1535 and TA1538 that has been accepted by the majority of the laboratories that conduct the Ames test (9). Plate counts from both test strains fall between this range; the background revertant frequencies characteristic of the UBC undergraduate laboratory thus conform to the historical spontaneous revertant frequency.

A high degree of variance was seen within each test condition on the triplicate plates (data not shown). This variance was likely due to the high inherent variation in revertant counts versus conventional plate counts. Another contributing factor may be due to stochasticity, slight differences in growth kinetics and physiology that are normally present in a cell line (6).

A non-statistical procedure was used to evaluate the results of the Ames test (9). From the results obtained in this study, n-C_{60} was classified as non-mutagenic in the specific TA1535 strain that was used and as inconclusive in the TA1538 strain. Specifically, n-C_{60} probably did not cause a transversion in *hisG46* of the TA1538 strain or a point deletion (frameshift) in *hisD3052 of* the TA1535 strain, genes needed for reconstitution of histidine synthesis (9).

The low number of revertants and absence of a dose-response relationship and hence the non-mutagenic response of n-C_{60}, may be accounted for, since the TA1535 strain used in this study retained a partial *uvrB* repair mechanism (data not shown).

The classification of n-C_{60} as either a mutagen or non-mutagen cannot be determined from this study, as a reproducible testing of at least 8 concentrations is required, according to recently published standards (9). Despite this technical hindrance, this study provides evidence for the classification of n-C_{60} as not a strong mutagen. These results do not seem to correlate with the *in silico* molecular dynamics simulations previously performed (14).

Theoretically, a single unmodified C_{60} fullerene molecule should be able to form strong hydrophobic (water-repellent) interactions with DNA strands in regions where structural integrity is compromised (nicks, thymidine dimers, etc) (14). This stable creation of a dsDNA-C_{60} hybrid to a single stranded gap could result in the blockage of the repair site from the cell's DNA damage repair machinery and perhaps eventually lead to mutagenesis. However, this computer simulation does not take into account the physical/chemical changes the C_{60} particles undergo during the most widely used protocol to suspend C_{60} into a stable aqueous colloidal dispersion (3). Past experiments have shown that C_{60} in water forms fine crystal aggregates with significant negative charge (3). The source of the charge is unknown, but is most likely from clathrate crystal formation and the tendency of hydroxyl ion adsorption (3). The limitation of the computer simulation by Zhao *et al*., then, is the lack of incorporation of the electronegative properties of n-C_{60} crystalline aggregates supported through experimental evidence.

Biologically, this discrepancy has a significant impact on the binding behavior to DNA and interaction with water molecules. Zhao's modeling predicts a strong hydrophobic interaction between DNA molecules and C_{60}, along with the excursion of water from this region. However, the reported aggregation and charge of n-C_{60} may alter its interaction with DNA.

Our data shows that our *S. typhimurium* strains TA1535 and TA1538 are able to grow and survive in an n-C_{60} environment (at concentrations of 0.04-0.53 mg/L) at a comparable growth rate to a non-n-C_{60} environment (data not shown). We were unable to observe statistically significant difference in colony numbers or colony diameter between the control group and the n-C_{60} treated group

This appears to contradict Zhao's prediction that C_{60} fullerenes have an adverse effect on the structure and stability of DNA molecules, which, intuitively, would have an effect on the growth efficiency. We observed no difference in colony size and morphology between control and n-C_{60} treated cells. However, this observation can be misleading, as there may be a decrease in effect of n-C_{60}, due to an increase in dilution with colony growth.

Although this study did not rule out the possibility that n-C_{60} could bind to and damage the DNA, the lack of clear distinction between n-C_{60} treated and untreated revertant numbers observed in this study provides evidence that n-C_{60} does not cause *detectable* damage to DNA that is not repairable by the SOS DNA repair mechanism and thus does not induce a detectable rate of mutagenesis.

However, our hypothesis, that n-C_{60} molecules lodge themselves into small DNA grooves or nicks and sterically hinder the SOS repair mechanism of the TA strains, cannot be proved nor disproved.

We were able to demonstrate that TA1538 and TA1535 are able to grow and survive in an n-C_{60} environment at a comparable efficiency to an n-C_{60} free environment; however we do not have experimental

evidence that the n-C_{60} permeated the bacterial membrane to associate with the cytosolic DNA.

In fact, the reported n-C_{60} aggregates may be repelled by the lipopolysaccharide (LPS) layer of the gram-negative enterobacter *Salmonella typhimurium* due to same charge repulsion, despite partial loss of LPS due to the *rfa* mutation in both our cell lines (8).

A consistent result was seen in almost all other studies to date: growth inhibition was seen in n-C_{60} intraperitoneally treated mice (9.5 mg/mouse) and intraveneously treated mice (13), in largemouth bass (0.5 mg/L of aquaria) (10), and in *E. coli* and *B. subtilis* exposed to ≥0.4 mg/L of n-C_{60} in the medium (8). The growth inhibition of the type Gram negative and positive strains that was previously seen at 0.4 mg/L of n-C_{60} (7) was not seen in this study. Growth inhibition can only be qualitatively observed by the thinning of the background lawns in the treated samples verses the negative control samples. The Ames test may have been too insensitive to detect the growth inhibition previously seen by a growth curve assay, as well as measured aerobic respiration rates (7).

The inability of n-C_{60} to cross the cellular membrane is supported by other studies (4, 8), which have shown n-C_{60} to interfere with cell surface electron transport chain members, but no cytoplasmic damage has been reported. Interestingly, the only effect of n-C_{60} that has been described thus far is a cytotoxic and not specifically a mutagenic effect. The observed toxicity is attributed to the lipid peroxidation of the membrane exclusively (10, 11). In addition, no evidence that the oxygen radicals generated by the n-C_{60} could act on DNA, protein or the mitochondria was found (11). It has been speculated that this activated oxygen radical conjugation to the n-C_{60} was due to light exposure. In this study, exposure of n-C_{60} to light was avoided wherever possible which may explain the absence of any growth inhibitory effects in contrast to those observed in previous studies (10, 11).

We are also unsure of the hydrodynamic diameter of the physical aggregates of n-C_{60}. This undoubtedly is important in whether or not the molecule can cross the bacterial membrane. There is a positive correlation between aggregate size and salt concentration (8). We used a final concentration of ~0.04 M phosphate buffered saline (PBS) in our experiments which may have increased the size of the n-C_{60} aggregates and prevented entry into cells.

Therefore, the hypothesis drawn from the results of this study is that n-C_{60} likely could not cross the plasma membrane and more importantly would not be able to cross the eukaryotic nuclear membrane. This implies the major effects of n-C_{60} on the cell concern mainly the interactions with the plasma membrane lipids.

FUTURE EXPERIMENTS

For future repetition of this experiment several aspects could be improved. Firstly a curve presenting the number of revertants as a function of the UV dosage delivered by the Chromatovue Stratalinker 2400 should be constructed. A dosage that induced a high rate of reversion, the number of revertants at least 2 fold and preferably at least 5 fold greater than the number of background revertants, for each tester strain should be selected as the positive control and as the dosage for the n-C_{60} and subsequent UV treated experimental samples that was attempted in this study. Secondly, to fortify the observation that n-C_{60} was not mutagenic at the concentrations tested, the sensitivity of the assay should be increased, by the utilization of a mammalian metabolic activator (e.g. Aroclor 1254 induced rate liver), the utilization of pKM101 containing TA strains (e.g. TA97, TA1538, TA1535, TA102 and TA104) and the utilization of strains that can detect different types of mutations (e.g. TA97 detects insertions that like TA1538 and TA1535 target GC sequences and especially TA102 and TA104 which detects transitions and transversions that are caused by mutagens that cause oxidative damage and that target TA sequences, in addition TA102 detects DNA cross-linking agents (8). Fourthly, the overnights for the phenotypic and the Ames tests should have been incubated for a shorter period of time. The cells in this study were incubated for ~22 hrs and were likely in stationary phase by the time they were plated. The strains TA1535 and TA1538 grown under aerobic conditions in semi-solid media reached stationary phase at ~16 hrs after the incubation start time (1). The stationary phase cells may have had different sensitivities to exposure to n-C_{60} and UV irradiation.

Before further experimentation on the potential health risks of n-C_{60} on bacteria is performed, whether n-C_{60} has a cytotoxic effect on bacteria and whether n-C_{60} can enter the cell and cause intracellular damage should be determined. A growth curve and parallel plate counts of the wild-type (LT2) and the various Ames strains exposed to varying concentrations of n-C_{60} should be obtained. The uptake of labeled fullerenes as a function of time should be performed (12). If research supports the fact that n-C_{60} cannot enter the cell, then the effect of n-C_{60} on the cell membrane should be elucidated. To test the physical disruption of the membrane a series of fluoresceinano-derivatized dextrans of different molecular weights could be incubated with the culture and n-C_{60} and the degree of intracellular fluorescence was measured (11). Alternatively the membrane potential of n-C_{60} treated and untreated bacterial cells may be compared to test the ability of n-C_{60} to damage the bacterial membrane.

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