Photochem. Photobiol. Vol. 36, pp. 517 to 523, 1982
Printed in Great Britain.

0031-8655/82/110517-07$03.00/0

MODE OF ACTION OF α-TERTHIENYL ON *ESCHERICHIA COLI*: EVIDENCE FOR A PHOTODYNAMIC EFFECT ON MEMBRANES

K. R. DOWNUM*, R. E. W. HANCOCK and G. H. N. TOWERS
Department of Botany and Microbiology, University of British Columbia, Vancouver, BC, Canada V6T 2B1

(*Received* 26 *March* 1982; *accepted* 24 *June* 1982)

Abstract—The photodynamic effects of α-terthienyl (αT) in near-UV light (UV-A) on *Escherichia coli* showed close agreement with the light absorption of αT at different wavelengths suggesting that αT is the primary absorbing molecule responsible for the photosensitized reaction. Studies with DNA repair deficient mutants of *E. coli* indicated that the bactericidal action of αT/UV-A was not mediated by DNA damage, in direct contrast to the well-known photosensitizer, 8-methoxypsoralen. By using a closed borosilicate glass reaction vessel and various gas mixtures, it was demonstrated that photosensitization of both *E. coli* and a more resistant bacterium, *Pseudomonas aeruginosa*, was absolutely dependent on the presence of oxygen. The rate of killing by αT/UV-A showed a rather small dependence on preincubation temperatures, with quite rapid killing at 5°C, suggesting that the movement of αT across the cytoplasmic membrane of *E. coli* is not the rate limiting step in killing and perhaps is not even necessary for killing. Sodium dodecyl sulphate-polyacrylamide gels of cell membrane proteins after 15 and 30 min of treatment with αT/UV-A showed substantial random crosslinking of these proteins. The results taken overall suggest that αT is a photodynamic photosensitizer which exerts its primary effect at the level of the cytoplasmic membrane.

INTRODUCTION

The photobiocidal effects associated with α-terthienyl (αT), a naturally occurring compound, have stimulated a great deal of interest in its toxic mechanism of action. α-Terthienyl is the only naturally derived thiophene to be examined in detail thus far, although several other natural and synthetic thiophenes have recently been found which also elicit 'phototoxic' antibiotic and nematocidal responses (Chan *et al.*, 1975; Gommers *et al.*, 1982). Differences of opinion regarding the mechanism of αT action exist in the literature. A non-photodynamic mechanism was proposed by Kagan *et al.* (1980) who reported that photosensitization of *Escherichia coli* was not oxygen dependent and that labelled αT covalently bonded to calf thymus DNA and *Candida utilis* DNA. In contrast, the photodynamic nature of αT was demonstrated in several other studies. Inactivation of the enzymes glucose-6-phosphate dehydrogenase (Bakker *et al.*, 1979) and acetylcholinesterase (Wat *et al.*, 1980) was found to require the presence of oxygen. In addition, photohemolysis of human erythrocytes (Wat *et al.*, 1980), killing of the nematode *Aphelenchus avenae* (Gommers *et al.*, 1980) and cellular inactivation of *E. coli* (Arnason *et al.*, 1981) were also shown to be oxygen dependent processes. Bakker *et al.* (1979) further demonstrated that glucose-6-phosphate dehydrogenase was protected by the presence of various singlet oxygen ($^1O_2$) quenchers and thus suggested that $^1O_2$, generated by photoactivated αT, was responsible for its toxic effects.

The *in vivo* mechanism of αT action was examined further in this study in the hope of clarifying some of the discrepancies pointed out in previous reports. The results clearly favour a photodynamic mechanism for αT and confirm the findings of Bakker *et al.* (1979), Wat *et al.* (1980) and Arnason *et al.* (1981). In addition, we report the effect of irradiation temperature on *E. coli* survival and evidence which suggests the involvement of membrane protein as a possible target of the photoactivated process.

MATERIALS AND METHODS

Irradiation sources. Three sources of UV-A irradiation (320–400 nm) were used during these studies. Fluorescent sources consisted of either four horizontal Sylvania F20T12/BLB lamps (7.2 W m^{-2} at 10 cm) or two vertical Sylvania F15T8/BL lamps (3.2 W m^{-2} at 15 cm). A 1000 W Xe arc lamp (Orion Corp.) was used to determine the action spectrum. Wavelength bands were selected at 10 nm intervals by interference filters (Corion Corp., half-bandwidth 10 nm). Irradiance of the fluorescent UV-A sources, as well as the wavelength specificity and irradiance of the interference filters used in conjunction with the Xe lamp, were measured with a Research Radiometer (International Light Inc.). Irradiance was determined at the wavelength maxima of each interference filter.

Bacterial preparation. The bacterial strains used in this study are listed in Table 1. Liquid cultures of *E. coli* B and *Pseudomonas aeruginosa* were grown at 37°C in an inorganic salt medium (BM2) containing 40 m*M* K_2HPO_4,

*Present address: T. H. Morgan School of Biological Sciences, University of Kentucky, Lexington, KY 40506, USA.

Table 1. Bacterial strains used during these studies and their relevant characteristics

Strain	Parent	Genotype	Source
Pseudomonas aeruginosa PA01	—	Wild	Dr. A. M. B. Kropinski
E. coli B	—	Wild	Laboratory stock
E. coli K12*			
AB 1157	—	Wild	Dr. A. J. Clark
JC 2926	AB 1157	*Rec* A^-	Dr. A. J. Clark
JC 3881	AB 1157	*Rec* A, B, C^-	Dr. A. J. Clark
JC 5519	AB 1157	*Rec* B, C^-	Dr. A. J. Clark
JC 5547	AB 1157	*Rec* B, C, F^-	Dr. A. J. Clark
JC 9239	AB 1157	*Rec* F^-	Dr. A. J. Clark

*All *E. coli* K12 strains used were isogenic derivatives of AB 1157 differing only by the indicated characteristics. The genotype of AB 1157 is F^-, *str*A-31, *tsx*-33, *sup*-37, *pro*A-2, *his*-4, *arg*E-3, *gal*K-2, *ara*-14, *xyl*-5, *mtl*-1, *thr*-1, *leu*-6, *thi*-1, *sup*E-44, *lac*Y-1, λ^-.

22 m*M* KH_2PO_4, 7 m*M* $(NH_4)_2SO_4$, 0.5 m*M* $MgSO_4$ and 10 μ*M* $FeSO_4$ with either 0.4% glucose or succinate added as the carbon source for *E. coli* and *P. aeruginosa*, respectively. All *P. aeruginosa* cultures also received KNO_3 as a terminal electron acceptor during anaerobic respiration. Subcultures in fresh medium were made to a final dilution of 1:100 for all experiments. Aerobic cultures were shaken at 250 rpm, while anaerobic cultures were grown in a BBL GasPak anaerobic system with GasPak O_2 combuster and CO_2 generator packs to create and maintain anaerobic conditions.

Assay procedure. A 3-necked, borosilicate reaction flask (multi-purpose jacketed reactor from Pierce Chemicals) with 10 mℓ capacity, equipped with stir bar, gas inlet and leur-lok sampling port, was used for all experiments unless otherwise indicated. Initially, 9 mℓ of BM2 medium with the appropriate supplements was added to the sterilized reaction flask. Either medical air (Linde, Type I), He (Linde, 99.995%) or He/O_2 (4:1), after being passed through a Millipore filter (Type HA, 0.45 μm) to prevent bacterial contamination, was bubbled through the growth medium for 15 min prior to addition of cells. After flushing with the appropriate gas, 1 mℓ of 1 in 10 diluted cells was added to the reaction flask. The cells were incubated for 1 h in air, 3 h in He or 30 min in He/O_2, during which periods of time viable counts indicated that the organisms were growing. Ten microliters of αT stock solution (1 mg mℓ^{-1} in 95% ethanol) was then added and the cultures were incubated for 30 min in the dark. Cells were then irradiated with continuous gas bubbling through the medium. Samples of 0.5 mℓ were withdrawn using a sterile 1 mℓ syringe at various times during incubation and irradiation. Viable cell numbers were determined by dilution and plating on enriched media followed by colony counting after 24 h at 37°C.

Action spectrum. These studies were carried out in sterile microtitration plates (Gibco Scientific) having 96 wells per plate. BM2 glucose medium (150 $\mu\ell$) was added to each of the wells. This was followed by the addition of approximately 10^3 cells prepared by diluting an overnight culture 100-fold into fresh BM2 medium. The sensitizer was then added (5 $\mu\ell$ of 1 mg mℓ^{-1} stock solution in 95% ethanol) to give a final concentration per well of 1 μg mℓ^{-1}. The plates were incubated at 37°C in the dark for 30 min and then irradiated. The irradiation source was a 1000 W Xe lamp (see Irradiation sources). The distance from the source was adjusted to give 7 W m^{-2} at each of the wavelengths. Following calibration, plates were irradiated at each wavelength for 2 min at room temperature and then incubated at 37°C for 18 h. A plate containing the sensitizer and bacteria, but unirradiated, was prepared as a dark control.

Bacterial growth was measured spectrophotometrically at 620 nm using a Titertek Multiscan photometer (Flow Laboratories). The optical density (OD) of the wells on each irradiated and control plate was determined immediately following irradiation and after 18 h incubation in the dark. Changes in OD were averaged to a representative change in OD. A ratio between OD's of irradiated and non-irradiated plates yielded a measure of the relative effectiveness of the fluence (sensitizer + irradiation). Dividing this value by the number of incident photons gave the relative photon efficiency at each wavelength. The data presented are averages of 3 experiments conducted on different days.

Rec *mutant assay.* All *rec*$^-$ strains were grown in nutrient broth (Difco) at 37°C with shaking at 250 rpm. Overnight cultures were diluted 1 in 100 and 0.1 mℓ was spread onto nutrient agar plates. Sterile Whatman No. 1 filter paper discs (7 mm) which had been previously loaded with αT (10 μg), bleomycin (15 μg; Bristol Laboratories) or 8-MOP (20 μg; Sigma) were placed onto the agar plates which were then incubated in the dark for 1 h at 37°C. Replicate plates were kept dark as controls, while the remaining plates were irradiated for 1 h by four Sylvania F20T12/BLB lamps. All plates were then incubated in the dark for 48 h at 37°C.

Temperature studies. E. coli B cells were inoculated into the reaction flask containing air saturated BM2 medium and incubated for 30 min at 37°C. Ten microliters of αT stock solution (1 mg mℓ^{-1} in 95% ethanol) was then added and the cells left in the dark for 30 min. The temperature of the culture was then adjusted to the desired irradiation temperature and allowed to equilibrate for 15 min prior to irradiation for 30 min.

The effect of incubation temperature on the photosensitized inactivation of *E. coli* by αT was investigated by lowering the culture temperature to 5°C for 15 min before the addition of αT. Following this prechill and 30 min incubation with the sensitizer at 5°C in the dark, the culture was irradiated at 5 and 37°C.

Crosslinking of E. coli *proteins.* Cultures of *E. coli* B were grown overnight to stationary phase at 37°C with shaking in BM2 containing 0.4% glucose. Two 10 mℓ portions were subcultured in 990 mℓ of fresh BM2 separately and grown to an OD of 0.6 at 520 nm. The cells were harvested by centrifugation at 8000 rpm for 10 min and resuspended in 100 mℓ of 10 m*M* phosphate buffer pH 7.0. The resulting suspension (approximately 3×10^9 cells mℓ^{-1}) was incubated with slow shaking for 15 min at 37°C prior to the addition of 1 μg mℓ^{-1} of αT in 95% ethanol. The suspension was then incubated for 30 min and subsequently irradiated by two horizontal Sylvania F15T8/BL lamps suspended 20 cm above the suspensions. Ten milliliter aliquots were removed prior to αT addition, after 30 min incubation with αT and following 15 and 30 min UV-A irradiation.

Viable cell counts were determined from each of the samples which were kept dark and on ice during the course of the experiment.

Cells were broken in a French press at 14000 psi after the addition of pancreatic DNase I (20 μg mℓ^{-1}; Sigma). The resulting broken cell suspensions were centrifuged at 10000 g for 15 min to remove the remaining intact cells. The suspension was then centrifuged at 180000 g for 1 h in a Beckman 50 Ti rotor. The pellet containing membranes and ribosomes was resuspended in 0.5 mℓ distilled water. The supernatant containing soluble or cytoplasmic proteins was precipitated with 0.5 M NaCl and twice the volume of ethanol (18 mℓ). The soluble proteins were collected after overnight precipitation at -20°C by centrifugation in an Eppendorf Model 54-12 centrifuge.

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Slab gel electrophoresis was used to analyze the soluble and membrane proteins of *E. coli* following irradiation in the presence of αT. The basic method was previously described by Lugtenberg *et al.* (1975). Separating or lower gels of 11% (vol/vol) acrylamide were prepared from a stock acrylamide solution containing 30% (wt/vol) acrylamide and 0.8% (wt/vol) N,N'-methylenebisacrylamide. The gels were polymerized by the addition (per 12.5 mℓ of gel mix) of 20 $\mu\ell$ of TEMED (N,N',N'-tetramethylethylenediamine) and 0.165 mℓ of 1% (wt/vol) ammonium persulfate. The stacking or upper gels were also prepared from the 30:0.8 acrylamide stock and were polymerized by the addition (per 5 mℓ of gel mix) of 10 $\mu\ell$ TEMED and 0.12 mℓ of 1% ammonium persulfate.

Protein samples were assayed by the method of Sandermann and Strominger (1972). Solubilization of proteins was accomplished by dilution of the sample in an equal volume of solubilization reduction mix containing 4% (wt/vol) SDS, 10% (vol/vol) 2-mercaptoethanol, 2-fold-concentrated upper gel buffer and 20% (vol/vol) glycerol followed by heating at 88°C for 10 min in a Temp-Block module heater (Lab Line Instruments Inc., Melrose Park, IL) filled with glycerol. Approximately 20 μg of protein was added per well. The gels were run for 3–3.5 h at a constant voltage of 150 V. The separated proteins were stained using Coomassie Brilliant Blue.

RESULTS

Action spectrum

The action spectrum for *E. coli* survival following treatment with αT and UV-A vs. the absorption spectrum for αT is shown in Fig. 1. Close agreement between the bactericidal activity and light absorption of the compound was observed. In both curves, the maximum was at 350 nm with declining effectiveness at shorter and longer wavelengths. These findings provide good evidence that αT, rather than a photoproduct formed during irradiation, is the primary absorbing molecule responsible for the photosensitized reaction.

Rec *mutant studies*

The inability of various mutants of *E. coli* to perform genetic recombination or post-replication repair was used to indicate whether DNA damage resulted from treatment by αT and UV-A. *Rec* mutants of *E. coli* K12 have much reduced ability to repair damaged sites on the DNA (Smith, 1977). Recombination mutants should show greater sensitivity to compounds which damage DNA than wild type cells which are capable of repair. This was, in fact, found to be the case in experiments with 8-MOP and bleomycin (Table 2), two compounds which have been shown to react with DNA. 8-MOP, which kills microorganisms via photoinduced monoadduct formation and interstrand crosslinkages with DNA (Song and Tapely, 1979), was selected as a photoactivatable control. This DNA intercalator inhibited *rec*$^-$ strains more that AB 1157, the wild type.

Bleomycin, which has been shown to cause the degradation of intracellular DNA (Onishi *et al.*, 1973), and to induce the synthesis of *rec* A protein in *E. coli* (Gudas and Pardee, 1976), was used as a non-photoactivated control. Greater zones of inhibition were also exhibited by the *rec*$^-$ strains when compared with *rec*$^+$ cells (AB 1157).

Both DNA repair deficient and wild type strains were equally sensitive to αT suggesting that the primary mode of action of αT is not damage to cellu-

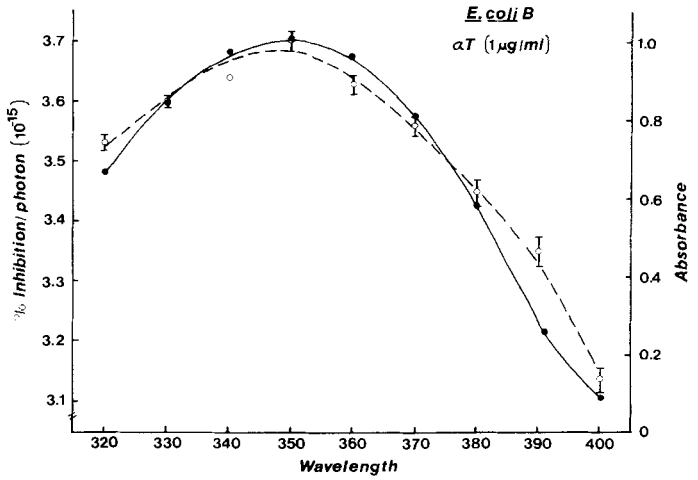

Figure 1. The photoinduced bactericidal action (○) on *E. coli* B (expressed as % absorbance at 620 nm of the culture per 10^{15} photons 18 h after αT/UV-A treatment) compared with the absorbance (●) of αT at different wavelengths between 320 and 400 nm.

Table 2. Response of various mutants of *E. coli* K12 to αT, 8-MOP and bleomycin irradiated for 60 min with UV-A

Strain	αT UV-A	8-MOP UV-A	Bleomycin UV-A	Bleomycin Dark
AB 1157	8.5 ± 0.9	3.0 ± 0.8	13.7 ± 1.2	14.0 ± 0.8
JC 2926 *recA*$^-$	10.3 ± 1.2	12.0 ± 0.8	24.7 ± 0.5	21.7 ± 0.9
JC 3881 *recABC*$^-$	9.3 ± 0.5	13.0 ± 0.0	25.7 ± 0.5	20.3 ± 2.5
JC 5519 *recBC*$^-$	9.0 ± 0.8	9.0 ± 0.8	21.7 ± 1.2	19.7 ± 1.2
JC 5547 *recBCF*$^-$	9.3 ± 0.9	8.3 ± 0.5	25.7 ± 2.1	17.7 ± 2.6
JC 9239 *recF*$^-$	7.7 ± 0.5	8.3 ± 0.5	17.2 ± 0.5	14.7 ± 0.5

Dark controls were also run but are not shown except for bleomycin treated cultures which caused inhibition in the dark. Inhibitory zones (from the edge of the disc to the edge of the inhibitory zone in mm) are followed by standard deviations.

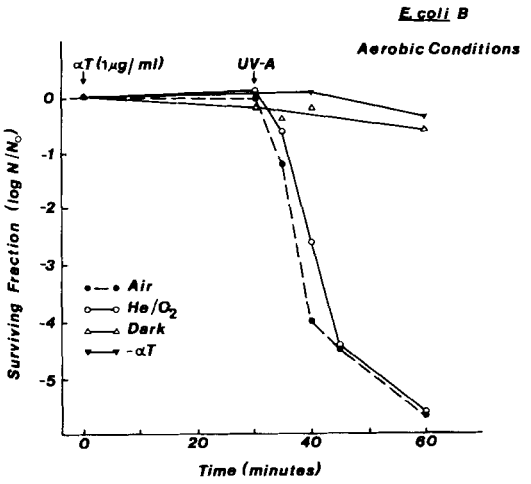

Figure 2. The survival of aerobically grown *E. coli* B irradiated with UV-A in the presence of αT under aerobic conditions. αT was added to cultures at 0 min and irradiation was started at 30 min.

lar DNA. No inhibition was found in response to any of the photoactivated compounds in the dark.

Aerobic–anaerobic studies

The photoinduced antibiotic activity of αT occurred only in the presence of air or oxygen and was independent of the respiratory status of cells just prior to irradiation. Cultures of *E. coli* grown and irradiated aerobically in the presence of αT showed a marked decrease in cell viability (Fig. 2). The replacement of air by a He/O_2 (4:1) mixture also allowed rapid killing (Fig. 2), suggesting that it was O_2, rather than a minor contaminant of air, which was required for killing by αT/UV-A. In contrast, cells respiring aerobically during preincubation in the presence of αT were protected from its effects by a switch from air to He prior to irradiation (Fig. 3). This suggests that the O_2 requirement was during the irradiation period rather than during the preincubation period.

Treatment of anaerobically grown cells which were irradiated under He caused no substantial decrease in cell survival (Fig. 3). Anaerobically grown cells which were transferred to an air atmosphere after the onset of irradiation, however, were subsequently killed in the presence of the sensitizer. There was a brief delay in the onset of killing which might have been related to the diffusion of oxygen through the suspension.

Higher doses of αT (2.5 μg $m/^{-1}$) were required to reduce the viability of *P. aeruginosa* (Fig. 4) to nearly the same extent as observed with *E. coli* (Fig. 2). Aerobic and anaerobic cultures of *P. aeruginosa* were supplemented with KNO_3, which functions in electron transport as an alternate electron acceptor in the absence of oxygen. Without KNO_3 *P. aeruginosa*, an aerobe, cannot grow under anaerobic conditions. We found that such anaerobically growing cultures were not substantially affected by irradiation in the presence of αT despite the existence of an anaerobic electron transport system. This is convincing evidence that oxygen is necessary for cellular inactivation as with *E. coli*.

Control experiments showed slight, but not always reproducible, effects of UV-A or αT alone on the survival of both *E. coli* and *P. aeruginosa* (Figs 2 and 4).

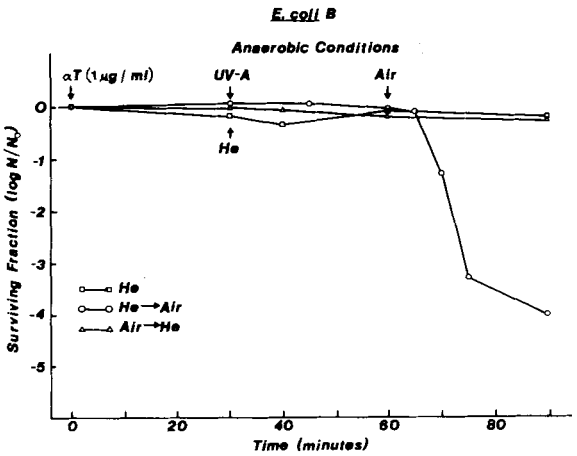

Figure 3. The survival of anaerobically grown *E. coli* B irradiated with UV-A in the presence of αT under aerobic and anaerobic conditions. αT was added to cultures at 0 min and irradiation was started at 30 min. Cells initially in He (○) were transferred to air at 60 min. Cells initially in air (△) were switched to He at 30 min.

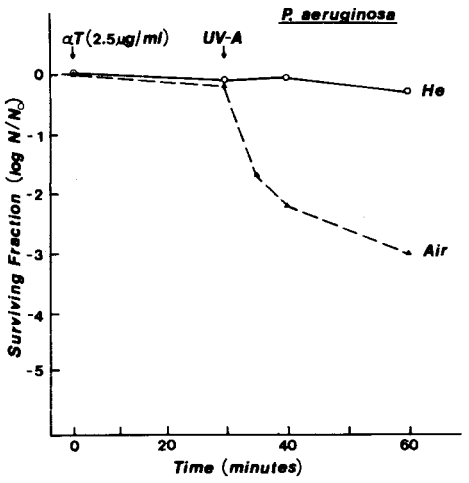

Figure 4. The survival of *P. aeruginosa* in the presence of αT and UV-A irradiation under aerobic (▲) and anaerobic (○) conditions. αT was added to cultures at 0 min and irradiation was started at 30 min. Control conditions of αT or UV-A alone caused no substantial *P. aeruginosa* death and are not shown.

These effects were very minor when compared to the effects noted above and were not considered further.

Temperature studies

An effect of irradiation temperature on the inactivation of *E. coli* by αT was observed (Fig. 5). Cells grown and incubated with the sensitizer at 37°C were subsequently irradiated at various temperatures between 5 and 42°C. Irradiation at 42°C following incubation resulted in immediate cellular inactivation (no shoulder) and high rates of killing. Reduced irradiation temperatures resulted in more pronounced lag or shoulder regions and slower killing rates. The greatest resistance to inactivation by αT was at 5°C. The apparent Arrhenius energy of activation was calculated to be approximately 6.5 kcal mol^{-1}. Rates of cellular inactivation during exponential killing at each irradiation temperature were used as the rate constants for this determination and were based on at least 3 experiments at each irradiation temperature.

Cultures incubated prior to irradiation at 5 or 37°C with αT and subsequently irradiated at 5°C had nearly identical survival curves (Fig. 6), even though the survival of cells incubated and irradiated at 37°C were substantially different than those incubated at 37°C and subsequently irradiated at 5°C (Fig. 5). Therefore, the incubation temperature does not seem to be critical for the photodynamic action of αT. This may indicate that the temperature effects observed in Fig. 5 do not result from inhibition of αT diffusion or transport across the plasma membrane at 5°C, otherwise the cells incubated at 37°C and irradiated at 5°C in Fig. 6 would more closely resemble the survival of cells incubated and irradiated at 37°C in Fig. 5. This suggests that the diffusion or transport of αT into the cell may not be necessary for inactivation to occur following irradiation, in agreement with the Arrhenius activation energy data and the substantial rate of killing at 5°C noted above.

Effects on cellular proteins

Figure 7 shows an electropherogram of membrane proteins from *E. coli* B before and after treatment with 1 μg $m\ell^{-1}$ αT and UV-A. The soluble proteins before addition and after 30 min dark incubation with the sensitizer showed no obvious differences, while proteins from irradiated cells showed reduced definition which was accompanied with general blurring of protein bands (data not shown). Membrane proteins showed the results of photosensitization even

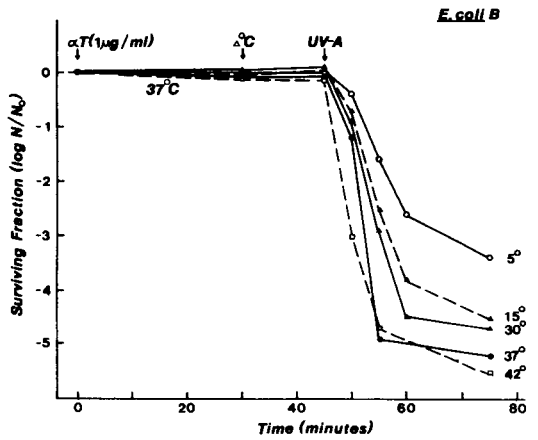

Figure 5. The survival of *E. coli* B in the presence of αT and UV-A irradiation at different irradiation temperatures between 5 and 42°C. All cultures were incubated with αT at 37°C for 30 min. The culture temperatures were then adjusted to the irradiation temperatures and allowed to equilibrate for 15 min prior to irradiation at 45 min.

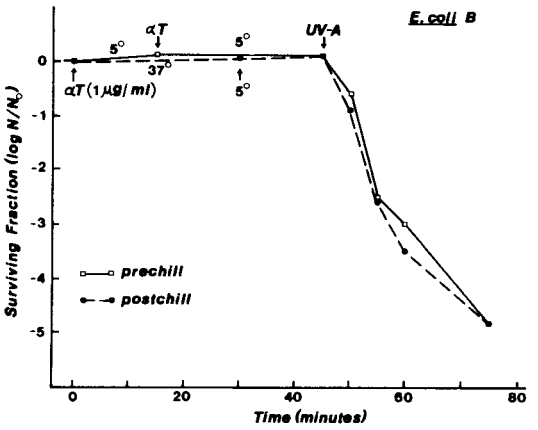

Figure 6. The effect of incubation temperature on the survival of *E. coli* B irradiated in the presence of αT at 5°C. Prechill treated cultures were first cooled to 5°C prior to adding αT and then incubated at 5°C for 30 min while postchill treated cultures were incubated with αT for 30 min and then cooled to 5°C for 15 min. Irradiation of all cultures was started at 45 min.

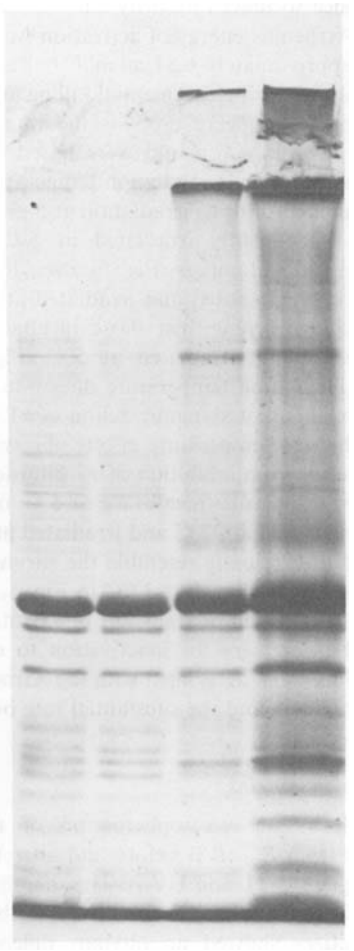

Figure 7. Sodium dodecyl sulfate-polyacrylamide gel electropherogram of the cell envelope membrane proteins prior to the addition of αT (lane A), after 30 min dark incubation with 1 $\mu g\ m\ell^{-1}$ αT (lane B) and after 15 (lane C) and 30 min (lane D) treatment with UV-A. Lane D has been about twofold overloaded in order to demonstrate the relative paucity of defined polypeptide bands. The protein aggregates at the top of the 3% stacking gel (SG) and the top of the 11% running gel (RG) as well as the outer membrane matrix protein (MP) are indicated.

more dramatically. No visible differences in proteins before addition and following 30 min dark incubation with 1 $\mu g\ m\ell^{-1}$ αT were evident (Fig. 7, lanes A and B). Following 15 (Fig. 7, lane C) and 30 (Fig. 7, lane D) min irradiation, the higher mol wt proteins were the most affected. General blurring of bands was found in addition to protein aggregation at the top of gels. A substantial amount of protein was found to have barely entered the stacking gel (3% acrylamide), suggesting the formation of protein complexes of at least one million daltons. These data strongly indicate random crosslinking leading to the formation of high mol wt aggregates of varying mol wt (appearing on the electrophoretogram as a blur rather than as defined polypeptide bands).

DISCUSSION

The requirement of oxygen for the expression of αT phototoxicity toward *E. coli* and *P. aeruginosa* has been clearly demonstrated in the present work which supports previous *in vitro* (Bakker *et al.*, 1979; Wat *et al.*, 1980) and *in vivo* (Gommers *et al.*, 1980; Arnason *et al.*, 1981) studies. The conflicting findings reported by Kagan *et al.* (1980) may be related to the test organism used in their study. *Candida utilis*, a eukaryote, might respond differently to photodynamic attack than the prokaryotes thus far studied. This possibility does not seem very likely, however, since Arnason *et al.* (1981) examined another yeast, *Saccharomyces cerevisiae*, and found no apparent differences in response compared to *E. coli*. This point, however, would seem to warrant further investigation.

Studies concerning the cellular target(s) of αT have also yielded conflicting data. MacRae *et al.* (1980a) were unable to detect chromosomal abberrations in cultured Syrian hamster cells following treatment with αT and UV-A. Kagan *et al.* (1980), on the other hand, reported that both calf thymus DNA *in vitro* and *C. utilis* DNA *in vivo* were targets of αT. In contrast, no evidence which would indicate that *E. coli* DNA was altered or damaged as a result of UV-A irradiation in the presence of αT was found in this study. It is conceivable that the high concentrations of αT (40 $\mu g\ m\ell^{-1}$), combined with the long irradiation times (2–4 h) used by Kagan's group, led to an increased likelihood of reaction with DNA. Alternatively, we have found that the organic synthesis of αT by the method of Kooreman and Wynberg (1967) yields impure compound based on analysis by high pressure liquid chromatography and gas chromatography–mass spectrometry. The low incorporation values reported by Kagan *et al.* could result from reaction of such impurities with DNA.

The substantial effect of αT and UV-A on inactivation of membrane-bound acetylcholinesterase of human erythrocytes (Yamamoto *et al.*, 1979) and on *E. coli* membrane proteins reported here demonstrates the importance of these membrane components as targets of photodynamic attack. Dubbelman *et al.* (1980) reported a remarkably similar result following irradiation of human erythrocytes in the presence of the photodynamic sensitizer protoporphyrin. They postulated that the blurring of protein bands and concurrent appearance of protein aggregates at the tops of polyacrylamide gels resulted from protein crosslinking following photooxidation of amino acid residues by $^1O_2$. However, they emphasize that the deterioration of membrane function, leading to increased cation permeability, is caused by photooxidation of amino acid residues rather than by crosslinking of membrane proteins. Further experiments with *E. coli* proteins would be important in determining whether crosslinking is indeed a critical factor.

Another line of evidence has also indicated the par-

ticipation of membranes in the photodynamic action of αT. MacRae *et al.* (1980b) demonstrated lesions in the cytoplasmic membranes of human erythrocytes irradiated in the presence of αT. This indication of membrane damage might reflect involvement of either the lipid or protein components of membranes or an interaction of the two.

The increase in *E. coli* survival when cells were irradiated below 42°C is a phenomenon which has been found previously in other *in vivo* studies with different sensitizers (Stenstrøm *et al.*, 1980; Wakayama *et al.*, 1980; Ito, 1981) and was postulated to be related to temperature dependent membrane fluidity. Indeed, Dubbelman *et al.* (1980) showed that crosslinking of erythrocyte membrane proteins was dependent on temperature, although they suggested that photooxidation of amino acid residues and enhanced cation permeability were virtually temperature independent. Our results demonstrate substantial rates of killing at 5°C (well below the mean transition temperature of 28.5°C for *E. coli* cells grown at 37°C; Nakayama *et al.*, 1980), a rather low Arrhenius activation energy, and no effect of preincubation at 5 or 37°C in the presence of αT on subsequent killing after UV-A irradiation. Thus, our results do not favour the concept that the temperature effects seen with αT are due to an inhibition of αT movement across the cytoplasmic membrane. This again argues against DNA as the cellular target. It may be that the rather mild temperature effects observed are due to the restriction of diffusion of O_2 or $^1O_2$. In future studies, we hope to investigate the relationship between temperature and the extent to which membrane proteins of *E. coli* are affected in greater detail.

Acknowledgements—We wish to acknowledge financial assistance from the National Scientific and Engineering Research Council of Canada to G.H.N.T. and to R.E.W.H. We also wish to thank Dr. E. Yamamoto for useful discussions.

REFERENCES

Arnason, T., G. F. Q. Chan, C.-K. Wat, K. Downum and G. H. N. Towers (1981) *Photochem. Photobiol.* **33,** 821–824.

Bakker, J., F. J. Gommers, I. Nieuwenhuis and H. Wynberg (1979) *J. Biol. Chem.* **254,** 1841–1844.

Chan, G. F. Q., G. H. N. Towers and J. C. Mitchell (1975) *Phytochemistry* **14,** 2295–2296.

Dubbelman, T. M. A. R., C. Haasnoot and J. van Steveninck (1980) *Biochim. Biophys. Acta* **601,** 220–227.

Gommers, F. J., J. Bakker and L. Smits (1980) *Nematologica* **26,** 369–375.

Gommers, F. J., J. Bakker and H. Wynberg (1982) *Photochem. Photobiol.* **35,** 615–619.

Gudas, L. J. and A. B. Pardee (1976) *J. Mol. Biol.* **101,** 459–477.

Ito, T. (1981) *Photochem. Photobiol.* **33,** 117–120.

Kagan, J., R. Gabriel and S. A. Reed (1980) *Photochem. Photobiol.* **31,** 465–469.

Kooreman, H. J. and H. Wynberg (1967) *Rec. Tr. Chim.* **86,** 37–43.

Lugtenberg, B., J. Meijers, R. Peters, P. vander Hook and L. van Alphen (1975) *FEBS Lett.* **58,** 254–258.

MacRae, W. D., G. F. Q. Chan, C.-K. Wat, G. H. N. Towers and J. Lam (1980a) *Experientia* **36,** 1096–1097.

MacRae, W. D., D. A. J. Irwin, T. Bisalputra and G. H. N. Towers (1980b) *Photobiochem. Photophysiol.* **1,** 309–318.

Nakayama, H., T. Mitsui, M. Nishihara and M. Kito (1980) *Biochim. Biophys. Acta* **601,** 1–10.

Onishi, T., K. Shimada and Y. Takagi (1973) *Biochim. Biophys. Acta* **312,** 248–258.

Sandermann, H. and J. L. Strominger (1972) *J. Biol. Chem.* **247,** 5123–5131.

Smith, K. C. (1977) In *The Science of Photobiology* (Edited by K. C. Smith), pp. 113–142. Plenum Press, New York.

Song, P.-S. and J. K. Tapely Jr. (1979) *Photochem. Photobiol.* **29,** 1177–1197.

Stenstrøm, A. G. K., J. Moan, G. Brunborg and T. Eklund (1980) *Photochem. Photobiol.* **32,** 349–352.

Wakayama, Y., M. Takagi and K. Yano (1980) *Photochem. Photobiol.* **32,** 601–605.

Wat, C.-K., W. D. MacRae, E. Yamamoto, G. H. N. Towers and J. Lam (1980) *Photochem. Photobiol.* **32,** 167–172.

Yamamoto, E., C.-K. Wat, W. D. MacRae and G. H. N. Towers (1979) *FEBS Lett.* **107,** 134–136.