11

EFFECTS OF SOME PHYSICO-CHEMICAL FACTORS ON ANTIMICROBIAL EFFICIENCY OF ULTRA VIOLET RADIATION

Dr. G.K. Konning, M.Pharm. PhD(Lond)
Dept. of Pharmaceutics
University of Science & Technology, Kumasi,Ghana

ABSTRACT

Ultraviolet radiation 254nm is highly inimical to microorganisms to the extent that substances which by themselves are mildly antagonistic offered no synergistic advantage when co-jointly used with the radiation. The course of sterilization by radiation was markedly influenced by the bulk of material. UV radiation 350nm on the other hand showed two distinctly opposing effects; a capacity to revitalize radiation-injured organism, albeit to a small extent, and a distinctly lethal effect; of the two, the latter effect is predominant.

Keywords: Ultra violet light, micro-organism, genome, polynucleotide, enzyme, reactivation.

INTRODUCTION

The influence of sunlight in minimizing the population of microorganisms in the atmosphere as a natural means of preventing spread of infectious diseases was common knowledge for many centuries. Similarly, many indigenous food preservation processes utilize sundrying to reduce moisture content thereby creating a degree of dehydration that would not favour survival of microbial contaminants present, but in so doing such processes also exploit the biocidal effect of sunlight. The inimical effect of sunlight on microorganisms is now firmly established as due mainly to its content of ultra violet (UV) light. Artificial sources of UV radiation in the form of commercial mercury lamps are now widely in use for controlling microbial contaminants of the atmosphere in enclosed areas e.g. hospital wards, surgical theatres, aseptic laboratories and in some food processing industries.

The UV spectrum ranges between wavelengths 400 to 200 nm approximately but the antagonistic effect of this broad spectrum is reported to vary with the wavelengths [1]. It is now generally accepted that optimum biocidal activity resides with wavelength 254nm. The mechanism by which UV destroys a living organism is not fully understood. It is, however believed that UV radiation affects cells by inducing lethal chemical changes in DNA or RNA or both, structures which are the repository of genetic information; changes in these nucleotides therefore ultimately impair cell replication, physiological function and account for most of the lethal effects, though disruption of protein structures e.g. enzymes might also be a contributory factor.

Much of the early research effort was directed primarily to the study of conditions such as would promote the revival of UV-injured organisms e.g. by modifying the chemical composition of recovery media or the physico-chemical conditions [2,3] that were considered essential for recovery of microorganisms. The general observation that UV 254nm is highly effective against microorganisms has led to the suggestion that in preparing killed bacterial or viral vaccines for active immunization for prophylactic purposes, UV radiation may have a theoretical advantage [1]. It is believed that since the genome (DNA) is more sensitive to UV damage than are the immunologically important surface antigens, the disease causing organism would be readily killed without much damage to its structures that actually induce antibody formation in a susceptible recipient - a necessary immunological even that must happen in order for the recipient host to acquire protection against the infection.

It is here considered that verification of the proposal might best be approached in stepwise investigations into some basic anti-microbial properties of UV 254nm and the probable influence, if any, that certain adjuvants of immunological products might exert. In the current work

therefore investigations were designed to study some of the factors as an integral part of the UV-microbial suspension system rather than as part of the recovery bacteriological medium.

MATERIALS AND METHODS

UV Lamp: Universal UV lamp CAMAG Type TL 900U; liberated UV light 254nm and 360nm, it was enclosed in a specially constructed box whose internal walls were blackened, a precaution adopted to obviate possible interference by extraneous light.

Test microorganisms: Escherichia coli (NCTC 5933, E. coli) and Staphylococcus aureus (NCTC 8765, Staph. aureus) were selected as biological indicators since these are common contaminants.

Bacteriological Media: Nutrient Broth, Nutrient Agar, McConkey Agar, MRVP and Mannitol Salt Agar, all Oxoid products; they were prepared, sterilized and stored according to Oxoid specification. McConkey Agar and MRVP were employed to establish the authenticity of the strain of E. coli used, and Mannitol Salt Agar, of the strain, Staph. aureus. All others were for routine purposes.

Overdried Nutrient Agar Plates: were used for recovery of organisms and these were prepared by drying freshly poured nutrient agar plates at 37°C for 24 hr; a drop of water delivered by a capillary dropping pipette onto the agar surface was absorbed within 20-30 mins.

Preparation of Stock Microbial Suspension: The authentic strain of organism was inoculated on agar slants and incubated at 37°C for 24 hr. The slants were washed with sterile water and pooled to form a stock suspension. A cycle of centrifuge-wash-centrifuge was adopted to free the organism of extraneous nutrient material which might protect the surface of the organism from radiation.

The suspension was finally standardised to establish its density (population per ml) using a procedure described by earlier workers [4]. Standardization made it feasible to calculate the volume of stock required to provide a desired density for all subsequent bacteriological reactions carried out. The stock was then stored at 4° until required for use.

EXPERIMENTAL

Intrinsic effect of UV 254nm on Micro organisms

A volume of standard bacterial suspension containing 6.0 x 10^3 viable organisms per ml of E. coli or Staph. aureus was pipetted into a wide mouthed plastic container. This was then positioned at a fixed (15cm) distance and at a definite geometry in relation to the UV source. The suspension was, then irradiated for a specified time. Aliquot samples of the treated bacterial suspension were immediately transferred onto overdried nutrient agar plates, allowed to diffuse and finally incubated at 37°C for 24 hr. Colonies that developed on the agar plate represented the number of surviving organisms in the aliquot samples after irradiation.

Effects of suspension bulk on efficiency of 254nm

A volume (0.5, 2.2, 4.9 or 8.6 ml) of the stock suspension containing 10^5 viables per ml was pipetted into each of four plastic containers of identical dimensions and made up to corresponding final volumes of 3, 6, 9 or 12ml, with sterile water. Each suspension was then positioned as before and irradiated at 254nm. The number of survivors was determined by the same method described above.

By the design arrangement used (i) the depth of suspension irradiated ranged from 3 to 12mm, (ii) the critical distance between UV source and the top layer of suspension (air distance) also varied from 147mm to 138mm; both factors, viz depth (bulk of suspension), and air distance were studied for their relative effects.

Tonicity effect on radiation performance

The standard inoculum of organism was suspended in hypo-, hyper, and isotonic solutions of sodium chloride. Each was immediately after preparation subjected to 254nm and the procedure for determining survivors followed as outlined above.

Response of 254nm-injured organisms to 350nm exposure

Reports that UV 350nm has the capacity to revitalize radiation damaged cells led to the design of this investigation. A standard test organisms was first irradiated at 254nm for a pre-determined time period equivalent to LT_{50} (30 secs) viz the time required to kill 50% approximately of the initial inoculum. A sample was withdrawn at LT_{50} and incubated while the remaining portion of suspension in the container was instantly divided into two portions and respectively switched to 350nm or transferred into dark storage. They were sampled at regular intervals, plated and the survivors determined,

with LT_{50} counts representing the 'initial'pupulation just prior to exposure at 350nm or dark storage.

RESULTS AND DISCUSSION

Intrinsic antibacterial activity of UV 254nm

The number of organisms surviving UV 254 nm treatment at specified periods of exposure for both *E. coli* and *Staph. aureus* were recorded (Table 1) but for the purposes of this work survivors were calculated as percentages of the initial inoculum. The relationship presented as log % survivors against time of exposure was found to be sigmoidal (Fig.1). The observed pattern was strikingly similar to that first reported by Chick [5] who used a chemical disinfecting agent, and for which he applied the classical equation:

$$\ln N = \ln N_o - KT$$

where N_o is the number of organisms prior to treatment at zero time; N, number surviving at any specified time (T) when the death rate is K. In the current work, N was expressed as percentage as indicated above.

TABLE 1: Effect of UV 254nm on Microbial Survival

Exposure Time Secs	No. Survivors ml^{-1} ($\times 10^5$)	
	E. coli	*Staph. aureus*
0	6.00	6.00
30	2.54	3.22
40	1.56	2.19
50	0.72	1.51
60	0.28	0.90
65		0.72
70	0.16	0.50

There was death initially of the relatively less resistant members of the population succeeded by the more resistant. In chemical disinfection the reason offered to explain the observed slow or rapid death was disparity in age of cells within the population. In the current context, however, it is considered that resistance may not necessarily be limited to age. In chemical disinfection, the organisms are nearly all completely surrounded by the disinfectant molecules which presumably have relatively

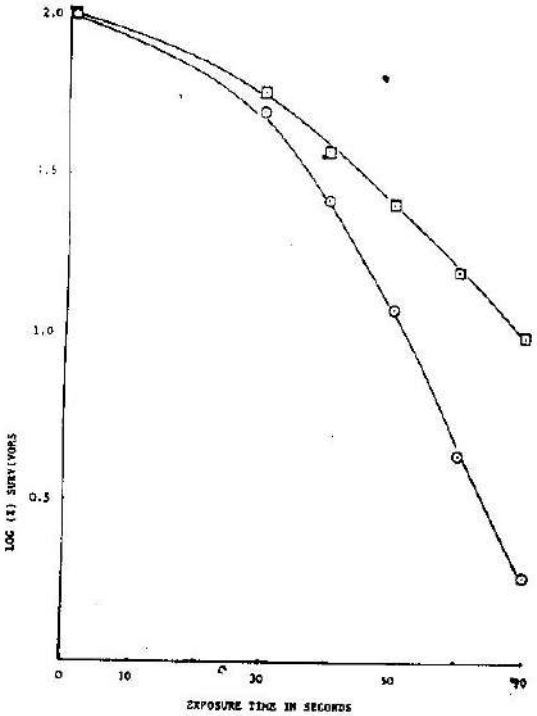

FIG. 1: Effect of UV 254nm radiation on survival of organisms
KEY: *E. coli* (○) *Staph. aureus* (□)

easy access to all target areas on the cell wall or in the cytoplasm. UV radiation on the other hand, is known to cause damage to cells by inducing cross-linking of bases on the genome (DNA). The time-dependent nature of the process (Fig.1) seems to suggest that a number of UV quanta of energy must necessarily hit the target sites on the DNA to produce effect. It is plausible that due to the folded three-dimensional structure of DNA, targets that are oriented in different geometrical positions in relation to the uniplanar flow of radiation, unlike chemical disinfectant, would be destroyed at different times - the more directly the target is orientated towards the source, the more rapidly damage can occur.

Depth of suspension on UV efficiency

The relationship between survivors and time was of similar pattern to that recorded earlier in Fig. 1. The data were computed as death rates (Table 2), using the equation above, to reflect influence of depth and bulk of suspension treated and the effective exposure distance. The distance between UV light source and the top layer of the most bulky suspension (12mm depth) was closer to the source than the smallest (3mm depth) yet proximity, contrary to expectation, offered no practical advantage. Indeed 254nm

TABLE 2: Influence of Suspension Depth on Biocidal Efficiency (death rates) of UV 254nm

Suspension depth (mm)	UV Distance mm	Death Rates (% Sec^{-1})	
		E. coli	Staph. aureus
3	147	0.092	0.040
6	144	0.087	0.040
9	141	0.084	0.033
12	138	0.075	0.029

was found to be more effective in reaching and killing a target cell in the smaller-bulk suspension which was further away from the source much more readily (death rate K = 0.092% sec.-1) than was possible in the more bulky (K = 0.075% sec^{-1}), for E. coli. A similar evidence was found for Staph. aureus.

The current observation appeared to indicate that distance traversed in the liquid mass had a far greater influence on diminishing the efficiency of UV radiation than did the same distance in air within the limits of the investigation.

Osmotic environment on susceptibility of organisms to UV

The lethal efficiency of UV 254nm as influenced by the osmotic environment was recorded and from the survivor-time curves obtained, death rates were calculated (Table 3). E. coli was found to be more susceptible than Staph. aureus a finding consistent with those presented above (Table 1, 2) in the absence of sodium chloride.

Osmotic solutions in combination with chemical disinfectants are known to be inimical to the survival of microorganisms [6] the effect being attributed to cell structure distortions that occur [7]. While Staph. aureus is more tolerant of variations in salt concentrations, E. coli is not, yet there was no evidence to suggest that the range covered - which reflects both hypo - and hypertonic solutions had any definitive effect on the antimicrobial activity of UV 254nm. The apparent failure of the osmotic factor to exert a synerstic effect is plausibly that while UV 254nm by itself destroys vegetative organisms in a time scale of seconds, the salt solutions are far less dramatic in their destructive capacity (>5 weeks). The significantly higher inherent biocidal effi-

TABLE 3: Activity (Death Rates) of UV 254nm Against Organisms Suspended in Sodium Chloride

Sodium Chloride % w/v	Death Rates (% Sec^{-1})	
	E. coli	Staph. aureus
0	0.090	0.040
0.1	090	0.036
0.5	084	0.037
0.9	0.082	0.041
1.5	0.089	0.035
2.0	0.086	0.036

ciency of 254nm must therefore account for the complete occlusion of possible contribution by the ionic environment.

Effect of UV 350nm on 254nm injured organisms

A population of organisms exposed to radiation at 254nm for a period within which 50% approximately is killed (LT_{50}) would contain in addition to the dead, moribund organisms at various stages of death. The results obtained when the damaged cells were subjected to 350nm radiation (Fig.2) indicated that there was the potential for revitalization of some injured cells as reflected by the increase in number. The work therefore collaborated the observation of an earlier work [8].

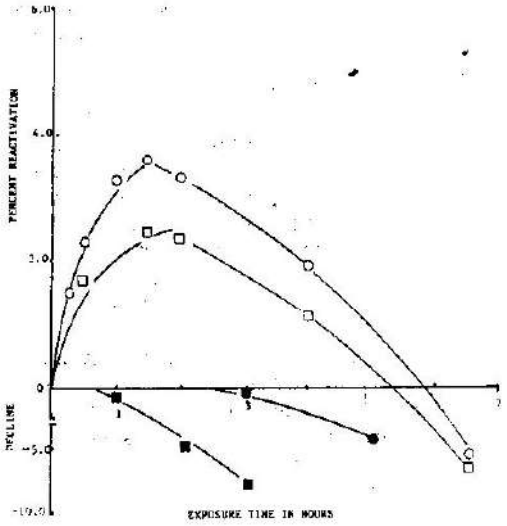

FIG.2: Influence of 350nm and darkness on organisms pre-exposed to 254nm.
KEY: E. coli (○) 350nm (●) Darkness
Staph. aureus (□) 350nm (■) Darkness

Reactivation of injured organisms subjected to 350nm radiation has been attributed to a repair mechanism that involves the synthesis and insertion of new nucleotides in place of damaged units by special enzymes e.g. DNA-synthetase, DNA-ligase etc [9,10]. The current work, however showed reactivation to be only a limited phenomenon. The maximum reactivation recorded was 2.4% for Staph aureus, and 3.5% for E. coli which suggests that less than 5% of the survivors had their polynucleotide structure (DNA) sufficiently restored by repair activity for them to undergo sustained growth and multiplication.

Further observation revealed that the limited reactivation of under 350nm treatment was short-lived as it was soon superseded by a definite and persistent decline. Persistent investigation subsequently established 350nm itself to be distinctly, though weakly, biocidal; its lethal efficiency in terms of death rate being 0.107% hr^{-1} for E. coli and 0.08% hr^{-1} for Staph aureus, theoretically, a relatively mild effect in comparison with that of 254nm.

The two observations namely revitalization and antagonism both associated with 350nm are apparently contradictory but an explanation may lie with the nature of the repair enzymes and of UV 350nm themselves. Repair enzymes would not ordinarily be present as a constitutive part of a normal intact cell; more likely, they are synthesized or activated from dormant precursors by the influence of UV 350nm as adaptive enzymes when an exigency of cell damage exists. Indeed, that only injured organisms subjected to 350nm as opposed to those in dark storage were the only ones revitalized seems sufficient evidence for suggesting a positive role for 350nm in stimulating repair enzymes, whether UV 350nm acting alone on healthy cells would accelerate DNA synthesis and therefore cell multiplication is not clear; it does however appear that even if it acts in both capacities simultaneously, its destructive effect would eventually prevail. This may offer a plausible reason for the brief period of revitalization and ultimate decline.

The continued death of injured organisms in dark storage is evidence that survivors, when irreversibly damaged would die off even in the absence of a lethal agent such as 350nm.

CONCLUSION

Exposed to ultra violet radiation (254nm) some organisms died quickly and others later. While a similar observation in chemical disinfection is usually attributed to resistance due to age, it is conceivable that in addition to the reason offered, accessibility of radiation to susceptible target points on a genome (DNA) which is convoluted may be significant.

Radiation was highly effective but its effectiveness tended to diminish rapidly with increase in bulk of material exposed. A practical advantage could be derived of this for sterile pharmaceutical dosage forms that are often presented in small packs such as vaccines and antitoxic sera; since time of contact would be brief with little or no heat generated that would adversely affect these heat-liable biological products, they would be sterilized safely and economically.

The high intrinsic sterilization activity of UV 254nm obliterated any contributory effect due to

an ionic environment that was co-jointly applied. It might in future be of interest to examine what effect the presence of known antimicrobial agent would have on the intrinsic activity of UV 254nm.

The reported resuscitation of radiation-injured cells by the application of ultra violet radiation 350nm was found to be only a limited phenomenon among a few survivors which had not been permanently damaged. Presumably enzymes that were elaborated to repair damaged target structures under the influence of 350nm did not match the mild but nonetheless antagonistic tendency of this same radiation. The import of this observation might probably be that vaccines containing living (modified) organisms e.g. Mycobacterium tuberculosis (Bacillus-Calmette-Guerin Vaccine, B.P.) stand no risk of reactivation on casual exposure to daylight during use.

References

1. *David B.D., Dulbecco R, Eisen, N.H., Ginsberg A.S. and Wood W.B. "Principles of Microbiology and Immunology", Harper and Row Publishers. New York [1968] pp.340*
2. *Alper, T. and Gillies, N.R. [1958]. Restoration of Escherichia coli strain after irradiation; its dependence on suboptimal growth conditions, J. Gen. Microbial. Vol. 18, 461*
3. *Weatherwax R.S. [1956]. Reactivation of ultra violet irradiated Escherichia coli. J. Bact. 72, 329.*
4. *Bean H.S., Konning G.H. and Malcolm S.M. [1969]. A model for the influence of emulsion formulation on the activity of Phenolic preservatives. J. Pharm. Phamacol. 21 Suppl., 173-181.*
5. *Chick H. [1908]. An investigation of the laws of disinfection J. Hyg., Camb, 8, 92.*
6. *Berry, H., Jensen E. and Saller E.K. [1938]. Sterilization of thermolabile substance in presence of bacteriocides, Quat. J. Pharm.XI, 729*
7. *Brown A.D. [1964]. Aspects of bacterial response to the ionic environment Bact. Rev. 28, 296.*
8. *Ricklis E. [1965]. Studies on mechanism of repair of UV irradiated viral and bacterial DNA in vitro and in vivo Can. J. Biochem 43 1202-18*
9. *Crossman, Brown A, Feldberg R. and Mahler I. [1975]. Enzymatic Repair of DNA. Ann. Rev. Biochem. 44, 19-43.*
10. *Bell G.H., Davidson J.N., and Emslie-Smith D. [1972]. Textbook of Physiology and Biochemistry 7th Edn. C. Tinling and Co. Ltd. London, 538-9.*