20
INFLUENCE OF SOME ADJUVANTS ON LETHAL ACTIVITY OF ULTRAVIOLET(254nm) RADIATION

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ABSTRACT

The lethal efficacy of UV 254nm radiation has been assessed against a common microbial organism in the presence of a wide variety of chemical compounds which by themselves are either antimicrobiologically inert (Polysorbate-80), mildly active (chloroquine phosphate) or distinctly active (phenol and chlorocresol). Irrespective of their antimicrobial status, every one of the adjuvants adversely affected the lethal efficacy of UV radiation. The underlying cause was found to be due principally to absorption.

Key words
Surfactant, bactericide, absorption, pathogen, efficacy, wavelength.

INTRODUCTION

Ultra violet (UV) radiation (254nm) has several practical biological applications but of particular importance is its use in environmental hygiene. The advantage that UV irradiation leaves no toxic after-effects makes the process predominantly, the first choice for routine control of microbial contaminants in enclosed areas including surgical operating theatres, place of public crowding, and rooms containing experimental animals.

Investigations have been conducted in recent times into the activity of UV radiation in areas other than environmental hygiene. The main thrust of such efforts has been towards the study of factors that may affect revitalisation of micro-organisms after radiation exposure; such factors as the chemical composition of the bacteria recovery media, and the optimum temperature at which the UV-injured organisms may be incubated [1-3]. These are mostly a study of factors that form an integral part of the general recovery profile.

Little interest appears to have been focused on conditions that specifically have a direct bearing on the bactericidal activity of UV radiation in its immediate environment of operation viz factors that constitute a part of the radiation + micro-organism interaction profile. The need for this alternative line of study is that in applying radiation as a means of sterilisation of liquid products, as against air sanitation, UV will invariably be acting in an environment that contains constituents other than the biological targets and such constituents may exert an influence. For instance, one possible area of application inferred from an earlier report [4] is the usage of UV radiation in the pharmaceutical industry for the production of vaccine of the type containing killed pathogens, a type that constitutes a major proportion of the entire vaccine group used for conferring immunological resistance.

The conventional method for killing bacteria pathogens in the course of vaccine production depends on an internationally recognised process of heating (56°C) with a bactericide (British Pharmacopoeia recommended procedure). The incorporation of a bactericidal preservative as an integral part of the formulation is a mandatory requirement serving both to facilitate heat sterilization and to prevent chance contamination of the vaccine during use. Thus, irrespective of the procedure for killing pathogens for vaccine production, it would be a necessary requirement to add a bactericide for preservation purposes.

In the current work the set objective was to assess the efficacy of UV 254nm radiation as the lethal agent for a system that simulated a vaccine containing non-active and active antibacterial additives as adjuvants, the current work serving as a sequel to an earlier work [6] in which simple systems without adjuvants were used as models.

MATERIALS AND METHODS

Ultra Violet Source: Universal germicidal lamp CAMAG Type TL, 900U with 88% of the emitted energy at 254nm (Manufacturer's Specification). The lamp was enclosed in a box with blackened interior walls as a precautionary measure against spurious light.

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Chemical adjuvants: Polysorbate 80 (Polyoxyethylene-20 sorbitan monoleate), A.R. Quality, SG1.08); chloroquine phosphate: white powder stored in amber coloured bottles with airtight closure; phenol: colourless deliquescent crystals dried over silica gel for 24 hr before use; chlorocresol, (p-chloro-m-cresol): crystals were stored as for phenol.

Solutions of the adjuvants were prepared in sterile water and stored in amber coloured bottles at 4°C; when required, appropriate aliquots were diluted to provide the desired concentrations.

Bacteriological Media: Nutrient agar (Oxoid CM1) prepared, sterilised and stored in accordance with Oxoid specification was used for propagation of the test organisms.

Freshly prepared nutrient agar plates were partially dried in an incubator for 24 hr. at 37°; this "overdried" agar plate has the advantage of being absorptive of small amounts of water from bacterial suspensions placed thereon, and thus of allowing colonies of viable organisms to grow as discrete colonies on the surface.

Test Micro-Organism: An authentic strain of Escherichia coli (E. coli NCTC 5933) was cultured on nutrient agar slopes and incubated for 24 hr. at 37°. The crop of organisms was washed off with sterile water and pooled to obtain a stock suspension. This was washed thrice by centrifugation to free the organisms of extraneous debris on the cell surface. It was finally resuspended in sterile water.

E. coli was selected as a biological indicator because it has been used by several workers in the area of irradiation study probably because it is fairly resistant and a common contaminants. [2,3].

EXPERIMENTAL

Standardisation of Bacterial Suspension

The method used by Bean et al [7] was adopted. Essentially graded aliquot of the stock suspension were made up to 10ml with sterile water and the optical densities measured on EEL Nephelometer. The 10ml dilutions were each enumerated for viable count. From a standard curve of optical densities and viable counts constructed, the volume of all subsequent stock suspensions required to provide a specific inoculum size of known 'viable cells ml^{-1} was readily determined.

General Procedure for Irradiation

The procedure for determining the performance of UV radiation (254nm) against the test organism suspended in the various chemical adjuvants was essentially the same for polysorbate 80, chloroquine phosphate, phenol and chlorocresol. Generally, aliquot of the adjuvants and the bacterial suspension were mixed in a wide-mounted plastic container to obtain a final population of 6×10^3 viable cells ml^{-1}. The container was then placed on a fixed platform in the box shielding the UV radiation source so that the geometric position of the container in relation to the UV source in all instances was a standard 15cm from the source. The sample was irradiated.

At specific time intervals samples of the irradiated system were withdrawn with calibrated dropper-pipettes with a standard delivery volume of 0.02 ml. A sufficient number was delivered on to the overdried nutrient agar plate and allowed to diffuse for 15 min. prior to final incubation at 37° for 24hr. Bacterial colonies that developed were enumerated as representing the survivors of the UV-radiation treatment.

The activity of the antimicrobial substances viz phenol or chlorocresol-carried over in all sampled volumes transferred to the incubation medium was adequately abolished by the moisture and agar content of the medium on account of the very marked influence that dilution has on phenolic compounds. Abolition of activity ensured that no UV-radiation injured survivors carried over in the sampled volume were subjected to phenol treatment beyond the sampling time specified.

RESULTS AND DISCUSSION

Discrete colony formations were recorded as survivors of the radiation treatment. To facilitate analysis, the exponential equation first proposed [8] and subsequently applied by others [9] in such dynamic studies was adopted:

$$\text{Log } N/N_O = -KT$$

where N_O = initial number of organisms, N = number of survivors at any specified time T, and K = killing rate. For convenience N/N_O was expressed as a percentage. The killing rate was calculated as a parameter to reflect the trend in efficacy of the lethal system and as a basis for comparative study.

Effect of Polysorbate 80 on Radiation Efficacy

The bactericidal efficacy of UV 254nm radiation against the target organisms suspended in polysorbate-80 ranging in concentration up to 2.0% w/v was recorded. Calculations based on the equation above showed that for concentrations up to 0.5% w/v, the activity of the system as reflected by the killing rate ranged narrowly between 0.070 and 0.067% sec^{-1}, an observation that appeared to suggest that the lower concentrations of the surfactant made virtually no impact on the efficacy of UV radiation.

Polysorbate-80, being a surface active agent, in solution, lowers surface tension and forms

micelles at a critical threshold (critical micelle concentration, CMC). Though it is itself microbiologically inactive, it is firmly established that by reducing surface tension, polysorbate-80 improves the performance of chemical agents against microbial organisms [10]. The current work therefore seems to underscore a unique difference between the mode of action of a chemical bactericide and that of UV radiation. The explanation for the difference in effect of the surfactant as indicated by the impact made on one and not the other lies probably with the nature of the two bactericidal agents themselves. The electromagnetic nature of UV light endows it a strong capacity for cell penetration that leads to a high level of lethal activity. In the case of the chemical antimicrobial agents, however, molecular adsorption and subsequent transfer across cell wall and cell membrane would be important pre-determinants to exerting bactericidal activity. Since an environment of reduced surface tension is reported [11] to enhance cell-bactericide contact, it is conceivable that low concentrations of the surfactant are likely to improve adsorption, molecular translocation and therefore antimicrobial activity of the chemical bactericide while the same surface phenomena may exert no discernible influence on radiation penetration.

It was significant to note that further increases in the amount of the surfactant from 0.5% w/v to 2.0% w/v caused a dramatic depression in the bactericidal efficacy of UV 254nm radiation as reflected by a sharp decline in the corresponding killing rates from 0.067 to 0.006% sec^{-1}. In similar studies surfactant present in concentrations exceeding the CMC were reported to diminish or completely abolish the activity of chemical antimicrobial agents [12]. Various reasons proferred for such an observation included one of interaction through hydrogen bonding between surfactant and the chemical bactericides, and an uptake of the bactericide by micelles; it was given that both mechanisms reduced the effective amount free and available for antimicrobial activity. While the loss in efficacy of radiation as observed in the current work may similarly be attributed to micelles, it is conjectured, however, that the mechanisms involved, in the case of radiation, may be entirely different.

Polysorbate-80 forms micelles at 1.0%w/v approximately. It is conceivable that the unique arrangement of micelles in solution as an early report indicates [13] may provide structures with intra-palisade spaces that may envelope and protectively shield target cells from direct radiation hits; even more significantly, the observation that polysorbate absorbed a proportion of UV 254nm radiation (discussed below) offers an additional plausible cause for the marked decline in the bactericidal activity of the radiation in the presence of the surfactant in concentrations higher than the CMC.

Influence of Chloroquine Phosphate on Radiation Activity

A preliminary evaluation showed chloroquine phosphate to be antagonistic to the test organism, E. coli although the activity expressed in terms of the bactericidal parameter, (phenol coefficient =0.1) as found in the current work, may at best be described as weak in relation to that of phenol (coefficient = 1.0) a compound generally considered as the standard of bactericidal efficacy.

The pattern of behaviour of UV radiation 254nm against the target organism in the presence of chloroquine phosphate at pH6 is graphically represented (Fig.1). The result of log % survivors against period of exposure was in every case a straight line. Considering the killing rate as a factor of efficacy, it was found, for illustration, that

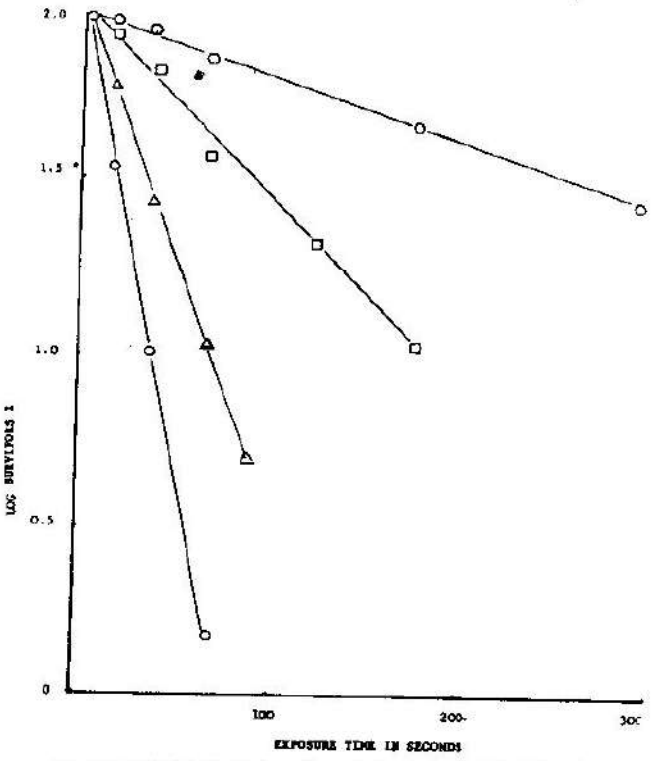

Fig.1: Effect of Chloroquine Phosphate non Lethal Activity of UV 254nm

CONC. O % w/v (O): 0.005% w/v (Δ) 0.010% w/v (□) 0.015% w/v (◇)

varying the concentration of chloroquine phosphate upwards to 0.015%w/v caused the bactericidal efficacy of the radiation to fall markedly from a rate of 0.061 to 0.003% sec^{-1}; similarly, at pH7, the same range of concentrations produced a downward change in efficacy from 0.064 to 0.004% sec^{-1} (Fig.2). Since the activities at pH6 and 7 were practically similar, the limited range of

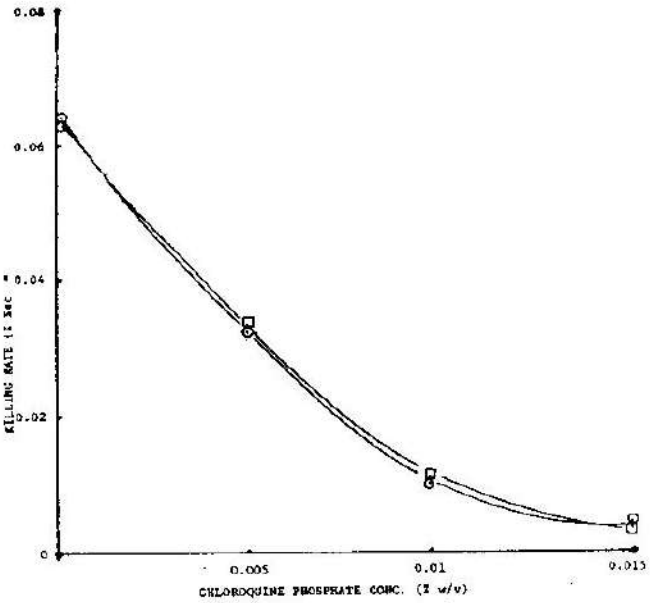

Fig. 2: Effect of Chloroquine Phosphate on the Activity of UV 254nm AT pH 6 (O), pH 7 (□)

pH used did not appear to affect chloroquine phosphate or the bactericidal activity of the system, any effects on radiation efficacy were therefore largely attributed to the influence exerted by chloroquine sulphate itself.

The overall result showed that the mild antimicrobial activity associated with chloroquine phosphate contributed nothing to enhance the efficacy of the radiation but rather adversely affected it; the adverse effect as in the case of polysorbate-80 was found to be linked with a physical interaction with radiation.

Effect of Phenol and Chlorocresol on Radiation Efficacy

The behaviour of UV 254nm in the presence of phenol in a joint interaction profile produced a pattern of results similar to that presented in Fig.1. By analysis all the concentrations of phenol 0.04%w/v to 0.7%w/v depressed radiation activity from a rate of 0.028 to 0.002 % sec^{-1}; similarly, an increase in chlorocresol concentration from 0.005%w/v to 0.06%w/v changed activity from 0.088 to 0.082% sec^{-1}. Thus basically both phenol and chlorocresol progressively diminished radiation efficacy. Two unique features arise out of the observations; viz

i. a decline in efficacy of UV radiation in the presence of another distinctly antimicrobial agent, and
ii. the relative margin of decline produced by phenol as compared to that by chlorocresol.

Both observations would seem on first considerations, to contradict theoretical projections.

When combinations of two or more antibiotics or combinations of bactericides are simultaneously applied, each component of the double lethal system exerts its antimicrobial activity towards the achievement of total activity that is superior to that obtained by the application of an individual component alone (synergistic effect), probably because each agent directs its attack on a different vulnerable part of the target cell and thus together produce a more rapid kill, or because one component sustains the attack longer due to relative differences in solubility rates. Combinations are, therefore, mutually advantageous. The dramatic losses in the lethal potency of the radiation in the presence of the phenolic compounds as recorded in the present work, however, suggest that any contributory effects of the bactericides towards destruction of the target organism appear to be largely obliterated by the UV + bactericide interaction.

The relative changes in efficacy of UV radiation 254 nm produced by the two phenolic compounds presented another significant feature. In real terms, an increase in chlorocresol concentration by a factor of 12 caused a decrease in efficacy of 2-fold approximately while an increase in phenol concentration by a factor of 18 produced as much as 11-fold decline in efficacy. The analysis indicates that the relatively more active chlorocresol produced only a marginal depression. The observation may appear to suggest that chlorocresol being comparatively more bactericidal (phenol coefficient = 10) has the capacity to potentiate the sustain the efficacy of radiation at a level that phenol, the less active analogue (phenol coefficient = 1.0) could not match. This basic presumption, however, would not explain why higher concentrations of chlorocresol failed to sustain radiation efficacy better than its lower concentrations. The levels of activity maintained by the radiation in the presence of the phenolic antimicrobial agents cannot therefore be accounted for solely by the inherent activities associated with the agents. The two observations outlined above for phenol and chlorocresol were found to have a common underlying feature similar to that for polysorbate-80 interaction through absorption as indicated below.

UV-Adjuvant Interactions

All the four adjuvants used were found to interact with UV-radiation absorbing at specific wavelengths; polysorbate-80 at a peak wavelength (λmax) of 236nm, chloroquine phosphate at 350nm, phenol at 270nm and chlorocresol at 280nm. Notwithstanding the characteristic absorption wavelength, each adjuvant absorbed significantly also at the specific bactericidal wavelength 254nm. Since absorption diminished

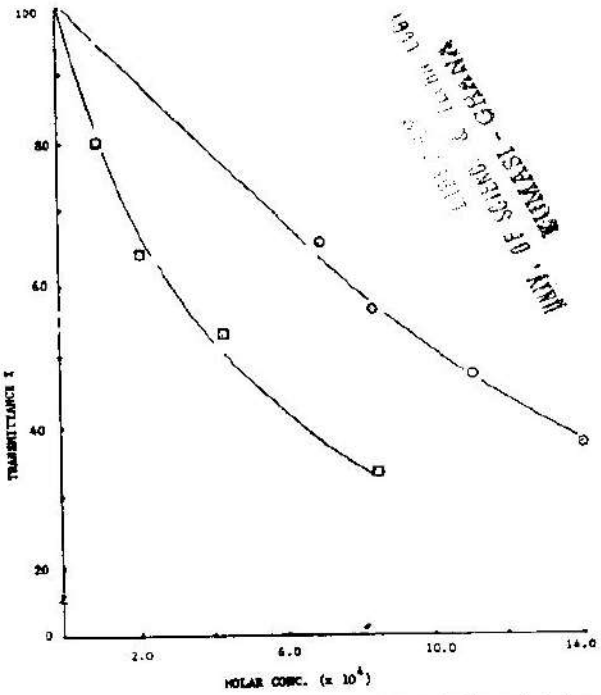

Fig. 3: Transmittance of uv 254mm Through Phenol (□) and Chloroscesol (○)

the amount of radiation transmitted as illustrated for phenol and chlorocresol (Fig.3) the quanta energy free for antimicrobial activity therefore diminished in progression with increases in adjuvant. The trend in decline in radiation efficacy as recorded in the presence of all four adjuvants as illustrated for chloroquine phosphate (Fig.2) is entirely consistent with the trend in the transmittance pattern.

The relative degree of depression of radiation effectiveness by phenol (11-fold) and chlorocresol (2-fold) as noted above appears to be typically illustrative of the influence of absorption. The absorption spectra showed that by its (λ max 270nm) relative proximity to the bactericidal radiation 254nm, phenol recorded a much stronger absorption than chlorocresol (λ max 280nm). Therefore, on the basis of stochiometric concentrations, chlorocresol absorbed a smaller proportion of 254nm and transmitted a greater fraction of the incident energy (Fig.3) for antimicrobial activity than did phenol. This observation appears to be a plausible reason for the observation that UV radiation 254nm maintained its efficacy far better in the presence of chlorocresol than in phenol.

CONCLUSION

An attempt to improve bacterial cell surface wetting and penetration in an environment of reduced surface tension such as occurs in solutions of low concentration (< CMC) of polysorbate-80 failed to advance the primary antimicrobial activity of UV radiation 254nm. Thus while surface phenomena play a role in molecular transfer they do not appear to have nearly the same effect on electromagnetic radiation whose cell penetration - dependent on its quanta energy as the driving force-could not readily be promoted on the basis of associated surface phenomena.

Higher amounts of the surfactant (> CMC) in the system significantly diminished the efficacy of radiation 254nm. A similar pattern of behaviour was manifested in every case when lethal radiation was applied conjointly with such other adjuvants as chloroquine phosphate, phenol or chlorocresol. While polysorbate 80 is bacteriologically inactive, phenol and chorocresol are on the contrary, intrinsically active. Therefore, the observation that the overall efficacy of phenol-UV or chlorocresol-UV lethal profiles was not synergistic but on the contrary antagonistic indicates that due consideration must be given to a number of factors when UV radiation is applied with the objective of achieving a set biological response.

In all of the cases cited the adverse effect of UV-adjuvant interaction was found to be associated with absorption. The total energy of radiation available to exert antimicrobial activity by the two phenolic analogues depended on the proximity of the specific wavelength of absorption of the compound to the lethal radiation 254nm. Thus phenol which relatively absorbed the greater proportion of radiation also produced the more dramatic decline in the efficacy of the lethal radiation with chlorocresol producing only a marginal effect.

Notwithstanding absorption and possible decline in efficacy of UV radiation, the residual sterilisation capacity of radiation 254nm nonetheless remained at a high speed level (in seconds). The current investigation appears to indicate that a judicious selection of parameters might not dramatically offset the efficacy of UV radiation as a sterilising agent in complex systems.

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