U-V INACTIVATION OF *BACILLUS SUBTILIS* SPORES ON POLYETHYLENE

BY

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A Thesis submitted to the Post-Graduate School, Ahmadu Bello University, in partial fulfilment of the requirements for the degree of Master of Science (Biophysics)

Department of Physics

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DECLARATION

I hereby declare that, the content of this thesis is an original work done by the author under the supervision of Professor (Dr.) J. Kiefer of the Institute of Biophysics, Strahlenzentrum, University of Giessen, West Germany.

To the best of my knowledge, no part of this work has been submitted anywhere for any academic award.

Works of others consulted in the text have been accordingly acknowledged in the reference.

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CERTIFICATION

This Thesis titled U-V inactivation of B.subtilis spores on Polyethylene by Samuel Effiong Ikpeme meets the regulations governing the award of the degree of M.Sc. Biophysics of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

Dedicated to GWB and the God of my Heart who makes all things possible.

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ABSTRACT

Bacillus subtilis 168 spores were used to study the sterilising effect of far ultraviolet radiation on polyethylene surfaces. The sterilising effect of heat as well as a combination of both heat and far UV radiation was also studied. Wet and air-dried samples of wild-type B subtilis 168 spores, placed on aluminium-coated polyethylene surfaces, were exposed to both heat and 254nm UV radiation. After exposure, they were recovered as suspensions in water and assayed for colony forming ability. The results show that high fluences of 254nm UV radiation produced high inactivation, but also tailed survival curves. It remains an open question as to the cause of this tail. Air-dried samples on polyethylene were found to be more sensitive than wet samples for low UV fluences. However, at high fluences, there was practically no difference in the sensitivity of the two samples. There was evidence of some degree of synergism between heat and UV light in the inactivation process.

TABLE OF CONTENTS

CHAPTER		PAGE
	Title Page	i
	Declaration	ii
	Certification	iii
	Dedication	iv
	Acknowledgement	v
	Abstract	vii
	Table of Contents	viii
	List of Tables	x
	List of Figures	xi
	Abbreviations	xiii
1.0	INTRODUCTION	1
2.0	Killing of Micro-organisms with UV ..	3
2.1	Rationale for the use of UV	3
2.2	Nature of UV radiation	5
2.3	Nature of UV damage to cells	9
2.4	Interaction of UV radiation with matter	99
2.5	Photochemistry of UV absorption	13
2.5.1	Inactivation and Cell Killing	18
2.5.2	Biological basis of Lethality	19
2.6	UV Sources	20
2.6.1	Natural Sources	20
2.6.2	Artificial Sources	20
2.7	Inactivation Kinetics	26
2.8	Bacterial Survival Curves	27

2.8.1	General description of UV survival curves	27
2.8.2	Kinetics of UV absorption	30
2.9	Effect of Heat on the Bacterial spore ..	33
2.9.1	Kinetics of Bacterial heat Inactivation	33
2.9.2	Multiple Critical sites theory	34
2.9.3	Innate Heterogeneity theory	35
2.10	Mechanism of action of heat and radiation	37
2.11	Repair of UV-induced damage	38
3.0	Materials and Methods	40
3.1	Stages in Sporulation	41
3.2	Cultivation of Spores	41
3.2.1	The Sporulation Medium	43
3.2.2	Growing and Harvesting of Spores	43
3.2.3	Cleaning of the Spores	44
4.3	Irradiation of Spores on Polyethylene ..	55
4.4	Effect of Heat on Spores	55
4.5	Effect of Fluence on Synergism	66
4.6	Synergism at 60°C	69
4.7	Prolonged Heating at 100°C	70
4.8	Combined Treatment at 85°C	71
4.9	Discussion	74
4.10	Conclusion	78
	References	80
	Appendix	87

LIST OF TABLES

		Page
2.1	Wavelength dependence of the % T<>T/T ratio of E. coli DNA in the steady state	16
2.2	Distribution of Pyr<>Pyr in DNA molecules of different base compositions at a fluence rate of 200Jm , λ = 265nm	16
2.3	Lethal effect of DNA damage of SV40 virus attributable to the disruption of individual genetic functions by 254nm UV-irradiation ...	38
4.1	Inactivation parameters for suspension irradiation	62
4.2	Parameters for UV-survival of spores on polyethylene	63
4.3	Inactivation parameters for survival of spores heated to 50°C	69
4.4	Parameters for spore survival at 85°C ...	74

LIST OF FIGURES

page

2.1 Similarity of the action spectrum for inactivation of E. coli cells, determined by F. L. Gates to the absorption spectrum of nucleic acids. 4

2.2(a) Action spectrum for the inactivation of B. subtilis spores 6

2.2(b) Absorption spectrum of DNA molecules and a typical protein 6

2.3(a) Spectrum of electromagnetic radiation on a logarithmic abscissa 7

2.3(b) UV, Visible and near Infrared on a linear abscissa 7

2.4(a) Electron transition through excitation by light absorption (JABLONSKI diagram) 11

2.4(b) Excitation energies of the first excited triplet states of DNA mononucluotides 11

2.5 Examples of DNA photoproducts formed in UV-irradiated cells 15

2.6 Photon fluence rate of the sun outside the atmosphere and at sea level with a zenith angle of 60°C 21

2.7 Emission spectrum of mercury lamps 24

2.8 Emission spectrum from gas tungsten arc welding 25

2.9	Typical curves for the inactivation of biologic organisms	28
2.10	Bacterial survival curves	31
3.1	Digramatic section through a bacterial spore	42
3.2	Technique for colony forming assay	50
3.3	Dilution series for 0 control	50
3.4	Calibration curve used for actinometry	54
4.1	Bacillus subtilis spores on polyethylene	60
4.2	Inactivation of B. subtilis spores in suspension	61
4.3	UV-survival of B. subtilis spores on polyethylene	64
4.4	Action of UV and heat on B. subtilis spores	65
4.5	Combined action of UV and heat on dry spores	67
4.6	UV-survival of mildly heated spores	68
4.7	Spore inactivation at 100°C as a function of time	70
4.8	Combined action of UV and heat at 85°C	72
4.9	Inactivation of spores at elevated UV fluences	73

ABBREVIATIONS

A	Adenine
C	Cytosine
C<>C	Cytosine dimer
DNA	Deoxyribonucleic Acid
E. coli	Escherichia coli
eV	Electron Volt
G	Guanine
λ	Wavelength
M	Molar
μl	Micro litre
N	Normal
Nm	Nanometer
Pyr	Pyrimidine
Pyr<>Pyr	Pyrimidine dimer
RNA	Ribonucleic Acid
RPM	Revolutions per minute
T	Thymine
T<>T	Thymine dimer
UV	Ultra Violet.

INTRODUCTION

The increased popularity, in recent years, of so-called "fast" foods has led to a search for better and more efficient methods for destruction of spoilage and pathogenic bacteria in packaging materials.

Mild heat treatment known as pasteurization, dates from the discoveries of Pasteur in 1860-64, and is widely used in the dairy industry, especially for asceptic packaging (Hsu, 1970; Voss, 1974; Cerf, 1976; Toledo et al.,1973). However, bacterial spores are extremely resistant to these methods (Swartling and Lindgren, 1968; Cerf and Hermier, 1972; Toledo et al., 1973; Ito et al., 1973). In 1979, Bayliss and Waites reported enhanced killing of bacterial spores by a combination of hydrogen peroxide and UV radiation.

In food related industries, however, factors such as the kind of food product itself, the quality of the packaging material or the technique of sealing may make these methods impossible or uneconomic.

In this work, far UV radiation (wavelength range100-280nm) is used to study the inactivation of bacterial spores placed on aluminium-coated polyethylene surfaces. An attempt is also made to study the inactivating effect of heat as well as any synergism between heat and far UV radiation. Polyethylene being used extensively in food-related industries for packing of their products, this work is in principle, a sterilisation study.The bacterial spore is a biological system which has been widely used in

radiation biology (Powers and Tallentire, 1968; Bcker et al., 1974; Horneck et al., 1984). Its special advantages comprise its great ease of storage, high resistance to environmental effects such as chemical substances, radiation and vacuum, and its synchronized cell cycle based on the absence of metabolism in the spore. Thus the dependence of the sensitivity to radiation on the cell cycle is eliminated (Kiefer, 1990). Metabolism and, thus, repair processes too can be reactivated synchronously in the spore population by incubation in nutrient medium.

The studies were carried out on bacterial spores belonging to the species Bacillus subtilis. It is known that, with the exception of Sarema lutea and Micrococus radiodurans, B. subtilis spores have the highest resistance to radiation and heat, of all bacteria. This fact is of particular importance in sterilisation studies because it implies that if B subtilis spores are killed by a particular process, then other species of bacteria, especially the food related ones like Clostridium botulium and Clostridium perfrigens, can be assumed killed.

In chapter two of this thesis, an attempt is made to present the theoretical background in chemistry and physics essential to an understanding of the biological effects. In the experiments, the effect of the surface (polyethylene), the irradiating state of the samples (whether wet or dry), the UV fluence rate as well as any possible synergism between heat and UV radiation will be examined.

CHAPTER TWO

2.1 Rationale for the use of UV for the killing of microorganisms

Action spectroscopy, a powerful tool in the search for the chromophores that may be associated with specific UV effects, have shown conclusively that far UV radiation has a high efficiency for inactivation of micro-organisms. Very important action spectra for killing of bacteria was obtained by Gates (1930), who reported spectra for killing of the bacteria E. coli and Staphilococus aureus. These spectra showed highest efficiency around 260-265nm, corresponding very closely to the absorption maxima of the nucleic acid bases (Burton, 1960) (see fig. 2.1). Emmons and Hollander, in 1939, showed that the action spectra for inactivation or mutation of the fungus, Trichphyton mentagrophytes, also showed a peak close to 260nm. Knapp et al., also in 1939, obtained a similar but sharper peak at 265nm for mutation of sperm of the liverwort, Sphaerocarpus donnelli. This pioneer work on action spectra was soon verified by other workers such as Hollander et al. (1940,1945) for Penicillin notatum and Neurospora crassa, Kaplan (1952) for Serratia marcescens, and their conclusions are now well established (Zelle and Hollaender, 1955). The maximum inactivation effect is reached in the range 250 to 270nm; for longer wavelengths, the inactivation of microorganisms is decreased and only minimal killing effect is obtained above 300nm (Bachman,

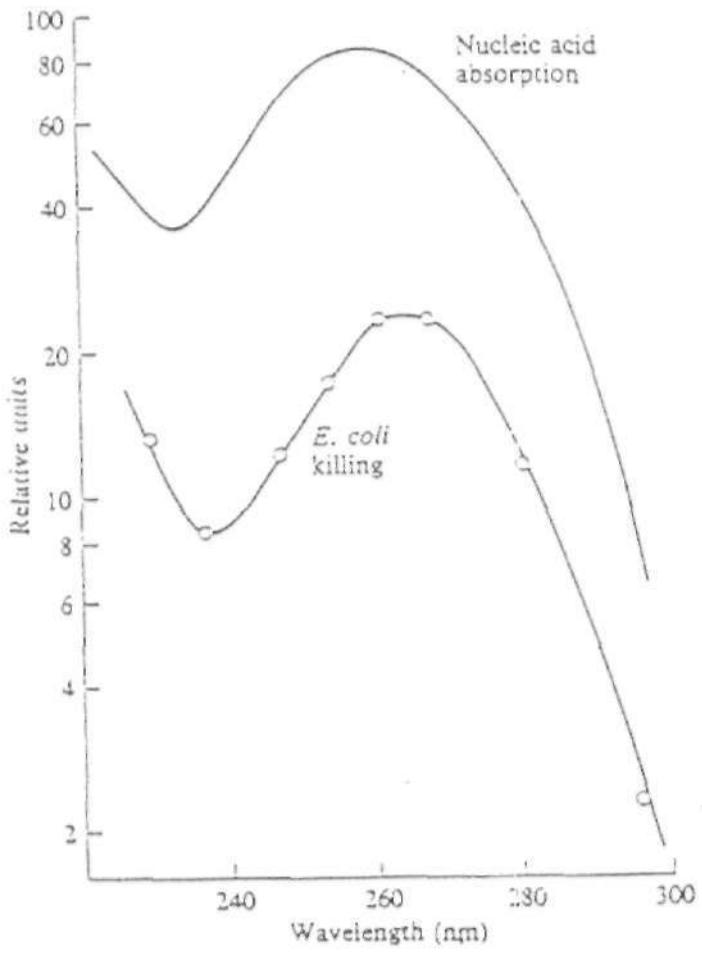


FIGURE 2.1: Similarity of the action spectrum for inactivation of E. coli cells, determined by F. L. Gates to the absorption spectrum of nucleic acids.
(Source: Rupert, 1960)

1976). One consequence of these investigations has been the development, and subsequent use, of the so-called GERMICIDAL lamp and STERILAMP which are, respectively, hot and cold cathode fluorescent tubes emitting almost exclusively 253.7nm radiation (Jagger, 1967). Recent work on spores of the bacteria Bacillus subtilis, by Lindberg et al. (1987),shows a result consistent with the findings referred to above (see figure 2.1).

2.2 The nature of UV radiation

Electromagnetic radiations are usually characterized by their wavelengths. In the range of UV and visible radiation, it is common to measure wavelengths in units of nanometers. Visible light lies in the range of 380 to 780nm. For biological purposes, it is convenient to regard the range of wavelengths from 100 to 400nm as constituting UV. The lower limit of 100nm is equivalent to photon energies of 12.4eV, which corresponds approximately to the limit for the production of ionization in biologically important materials. At the other end, the limit is the shortest visible wavelength; this varies from individual to individual, but lies between 380 and 400nm.

Because of differences in physical properties and biological effects, the UV region has been subdivided. Wavelengths shorter than approximately 190nm constitute the region of vacuum UV. This radiation is absorbed by air and water to such an extent that no biological effects can occur, unless very powerfull sources are used or the irradiation is carried out in vacuum (Harm, 1980). The

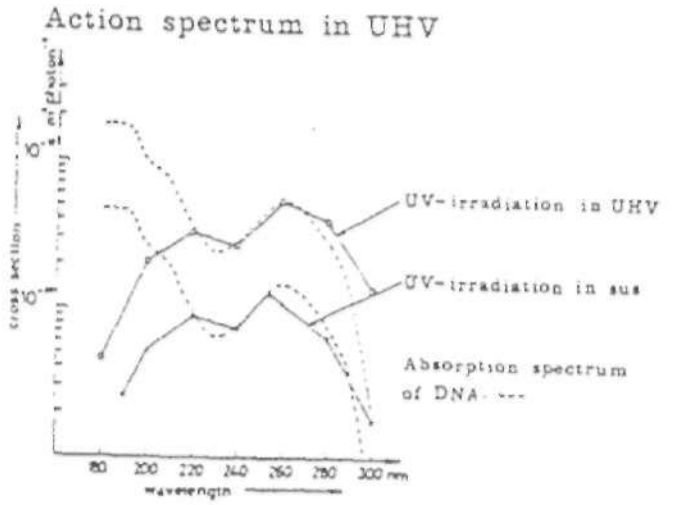


FIGURE 2.2(a): Action spectrum for the inactivation of B. subtilis spores.
(Source: Lindberg et al., 1985)

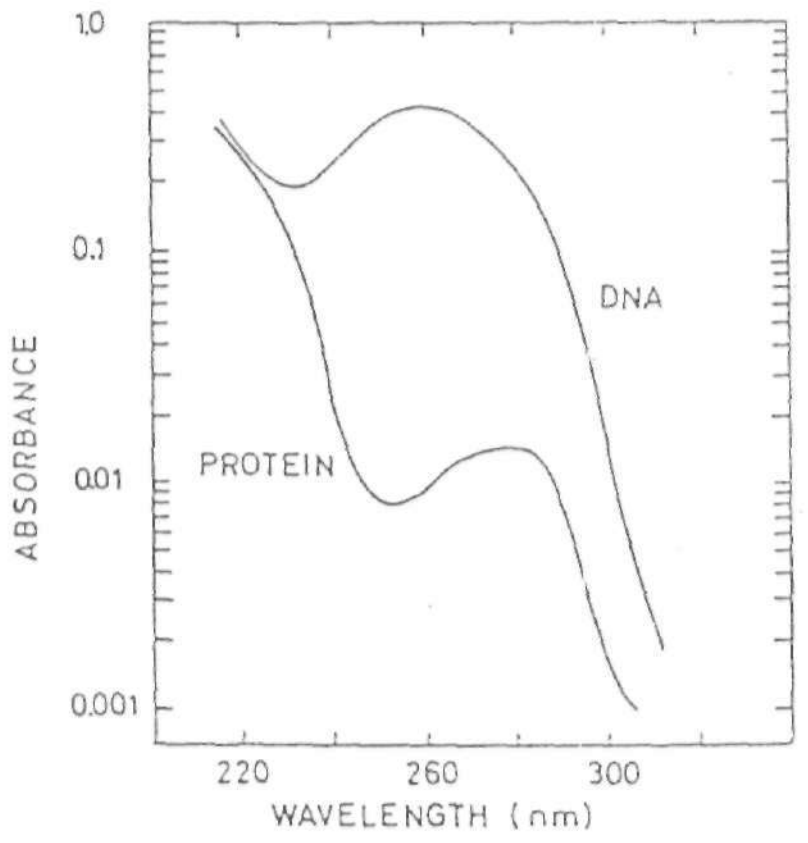


FIGURE 2.2(b): Absorption spectrum of DNA molecules and a typical protein (bovine serum albumin)
(Source: Harm, 1980)

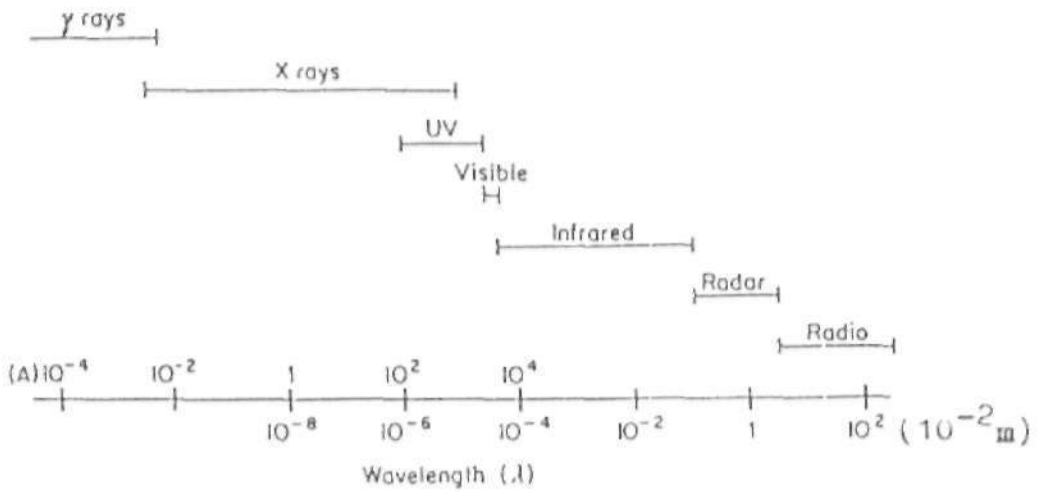


FIGURE 2.3(a): Spectrum of electromagnetic radiation on a logarithmic abscissa (*Source: Jagger, 1967*)

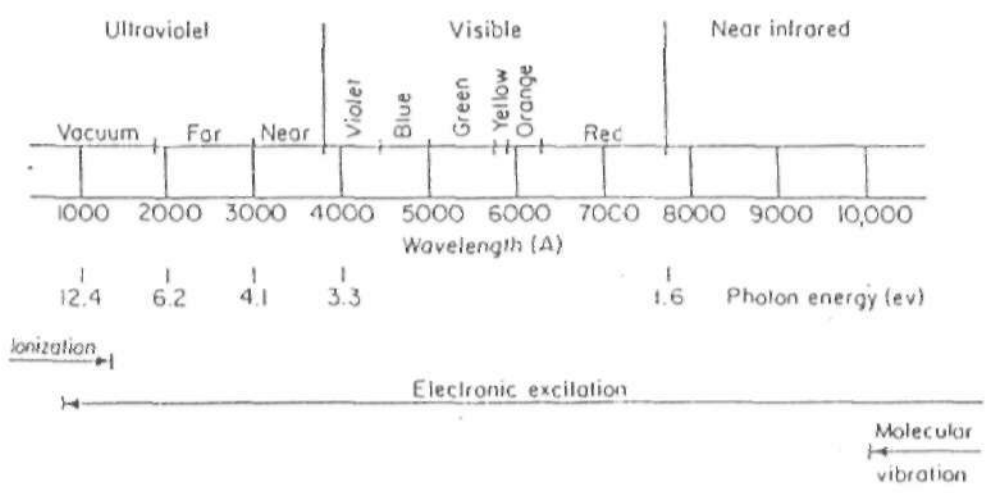


FIGURE 2.3(b): UV, Visible and near Infrared on a linear abscissa (Source: Jagger,1967)

remainder is then divided into the far-UV region between 190 and 300nm and the near-UV region between 300 and 400nm.

A different way of dividing up the UV region takes some of the biological effects into consideration. In this arrangement, the range 400-315nm, the so-called "black light" region is called UV-A. In this wavelength region, fluorescence can be induced in many substances. UV-B covers the range 315-280nm (the skin erythemal region). Most of the biologically active and potentially harmfull UV from the sun reaching the surface of the earth comes within this spectral region. UV-C includes radiation of wavelength less than 280nm (the germicidal region). These divisions are, however, arbitrary and usage varies from one worker to another (Faber, 1982).

Far UV does not ionize atoms and molecules as X-rays and particulate radiations do, but loses energy to matter in form of electronic excitations. Such excitations in the UV region can only occur in conjugated systems (alternating single and double bonds in a carbon chain) such as the benzene ring; it usually does not occur in single atoms or in highly saturated molecules such as carbohydrates and lipids. Therefore the absorption of UV in biologic material occurs primarily in nucleic acids, proteins or coenzymes, which usually possess conjugated structures. Also, unlike X-rays and the particulate radiations, once a UV photon has been absorbed, it disappears and does not give rise to further radiation. Therefore, UV does not penetrate far into biologic material and most of it is absorbed in the first ten microns.

2.3 The nature of UV damage to cells.

For biologic systems under normal physiologic conditions, there is very little similarity in the damages produced by UV compared with those produced by ionizing radiation. The most obvious reason for this lies in the observation that the oxygen effect (which influences about two-thirds of X-ray damage) does not occur with UV, and photoreactivation (which influences about two-thirds of UV damage), does not occur with X-rays (Jagger,1967).

Some of the action of far UV radiation on large cells may be due to absorption in proteins or coenzymes. However, in bacteria, which far UV above 220nm can easily penetrate as well as in many cells of higher organisms, nucleic acid is, overwhelmingly, the most important target molecule for biologic effects commonly studied, such as mutation and killing. This is because the genetic material of cells is unique and, therefore, crucially important to the cell (Harm,1980).

2.4 Interaction of UV radiation with matter

In general, photons of optical radiation can only be absorbed by atoms or molecules if the energy difference between the two states (dE) matches the energy content of the quantum (hf), that is:

$$dE = hf. \qquad (2.1)$$

where h = Planck's Constant and f = the frequency of radiation

Electron levels in molecules are superimposed by vibrational and rotational states whose energy differences are considerably smaller. Absorption normally occurs in the electronic ground state, that is, in the lowest possible one , which does not exclude that vibrational and/or rotational states may be excited as a result of thermal interactions. For photochemical processes, the first excited electronic state is the most important. In principle, excitations to higher excited states are possible, and do in fact occur, but their lifetime is so short that there is no chance for further reactions. They are rapidly deactivated to the first excited state via interactions within the molecule or with the environment. The lifetime of the first excited state is considerably longer so that photochemical processes may take place (Kiefer, 1990).

The excitation energy provided by UV photons is much higher than the energy of thermal motion of the molecules at physiological temperatures. The latter is of the order of Boltmann's constant times the absolute temperature, which, at 27°C amounts to only 0.026eV/molecule in contrast to the 3.3-6.5eV/molecule available from UV absorption.

The lifetime of a molecule in its usual excited state (10^{-10} to 10^{-8} sec), which is quite long compared with the time required for the energy absorption itself (approximately 10 sec), can be greatly extended if the excited electron is trapped in an (energetically lower) triplet state. In contrast to the usual singlet state, the

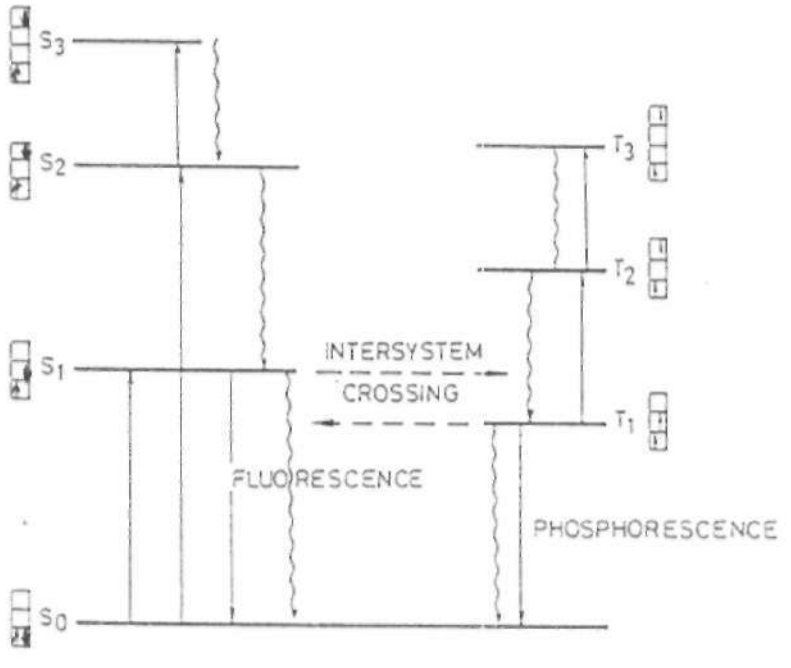


FIGURE 2.4(a): Electron transition through excitation by light absorption (JABLONSKI diagram) (Source: Kiefer, 1990)

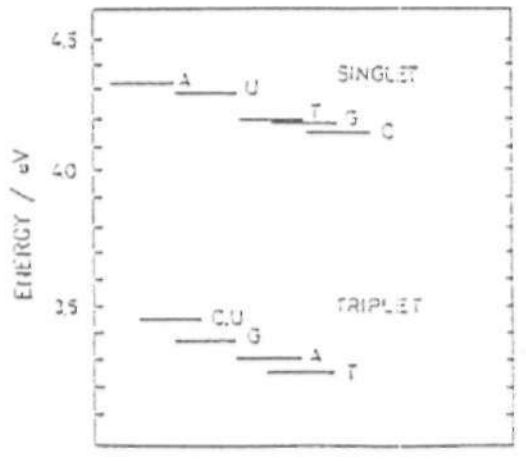


FIGURE 2.4(b): Excitation energies of the first excited triplet states of DNA mononucleotides

triplet state is characterized by two electrons with unpaired spins. Because the return to the ground state from the triplet is "forbidden" (that is, occurs at a low probability), the triplet state may last 10^{-3} sec or even longer and is, therefore, called metastable. (see fig. 2.4) As an excited electron returns to a lower energetic state, its excess energy may be disposed of in several ways :

(i)It can be emitted as a photon, resulting in fluorescence (fluorescent light is recognized by its usually longer wavelength, compared with the exciting radiation). Emission from molecules in the metastable excited state occurs over a longer period of time and is called phosphorescence.

(ii) The excitation energy can be dissipated as thermal energy in the course of collisions with other molecules.

(iii). The energy may cause the excited molecule to undergo a photochemical reaction that otherwise would not occur. The likelihood for this to happen increases with the lifetime of the excited state and is thus greatly enhanced for the triplet state. Photochenical reactions are the immediate effects of UV radiation in biologically relevant molecules, and constitutes the basis for the observed photobiological phenomena.

As has been stated earlier, the biological effectiveness of UV radiation is due primarily to its absorption in nucleic acids. The absorbing component within the nucleic acids are the nucleotide bases, in DNA usually the purine derivatives adenine and guanine, and the pyrimidine derivatives thymine and cytosine. RNA usually

contains uracil instead of thymine. Since nucleic acids from a variety of organisms may show considerable differences in their base compositions, not all absorption spectra of nucleic acids are alike. Nevertheless, their common features are an absorption maximum in the 260-265nm region and a rapid decline toward the longer wavelengths.

The absorption characteristics of RNA resemble those of DNA. However, alterations in RNA are usually of lesser biological consequence than similar alterations in DNA, because of the uniqueness of the latter as the genetic material. RNA, in the form of either messenger or transfer RNA is present in many copies and can be replaced as long as information for their production, carried by the DNA, is unaffected. Possible exceptions are those biological systems in which RNA is the only genetic material, as in some viruses and a few bacteriophages (Harm, 1980) .

The sugar-phosphate backbone of the DNA starts to absorb only at much shorter wavelengths (< 210nm). This fact is of little relevance in the present context, because, for sterilization purposes, UV light of 220-240nm is usually employed (von Sonntag, 1988).

2.5. Photochemistry of UV Absorption

As mentioned above, UV exposure in the wavelength range of 200 to 300nm affects only the bases in the DNA, the sugar moeities are not, at least, directly altered. Further, the purines are about ten times less sensitive to UV compared to pyrimidines so that, essentially, only the pyrimidines need be considered. Consequently, the far UV

photobiology of small cells is concerned primarily with effects on the pyrimidines of the DNA. Under physiologic conditions, the most important UV photoproduct is the pyrimidine dimer. It occurs in a reaction in which two adjacent pyrimidines in the same DNA strand form covalent bonds at their 5,6 positions, thus producing a cyclobutane ring (Kiefer, 1990).

$$\text{Pyr..Pyr} \underset{\text{h}\nu}{\overset{\text{h}\nu}{\rightleftharpoons}} \text{Pyr<>Pyr} \qquad (2.2)$$

The photochemical dimer formation is reversible . In the Pyr<>Pyr dimer, the 5,6 double bond of the pyrimidines is lost, hence it can no longer absorb strongly in the 260nm region. It absorbs significantly, however, at shorter wavelengths where the dimer can be split, thus allowing the integrity of the DNA to be regenerated (Patrick and Rahn,1977). Eventually, a steady state (k) is reached which depends on the wavelength of the incident light :

$$K = \frac{(\Phi_1)_\lambda \, (\text{Pyr..Pyr})}{(\Phi_2)_\lambda \cdot \epsilon(\text{Pyr<>Pyr})_\lambda} \qquad (2.3)$$

where ϵ is the extinction coefficient

Φ_1 and Φ_2 are, respectively, the quantum yields for the forward and backward reactions .

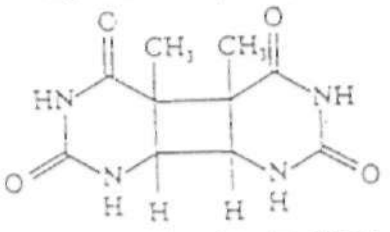
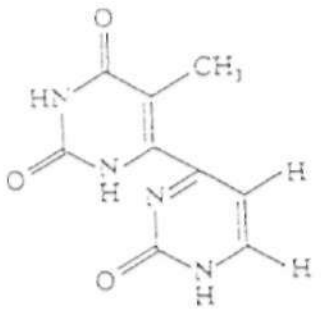
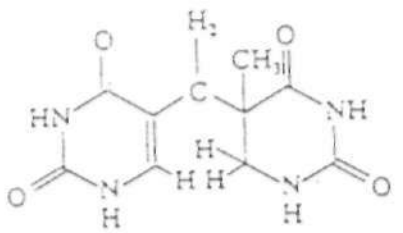
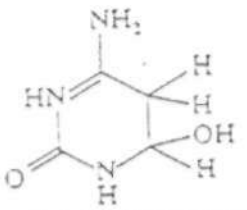
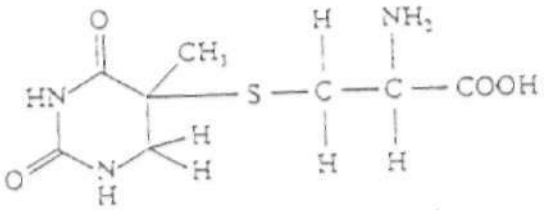
Class of photoproduct	*Representative photoproduct*
Cyclobutane-type dimers	 cys-syn thymine-thymine dimer
Pyrimidine adducts	 6–4′-(pyrimidin-2′-one) pyrimidine
Spore photoproduct	 5-thyminyl-5,6-dihydrothymine
Pyrimidine hydrates	 6-hydroxy-5,6-dihydrocytosine
DNA-protein crosslinks	 5-*S*-cysteine-5,6-dihydrothymine

FIGURE 2.5: Examples of DNA photoproducts formed in UV-irradiated cells
(Source: Harm, 1980)

The quantum yield of the forward reaction is of the order of 0.02. The efficiency of the reverse reaction is much higher, $\Phi_1 \approx$ 0.8 (Table 2.1 shows the ratio of T<>T over T in E. coli DNA at different wavelengths. The ratio of the various pyrimidine dimers depends mainly on the A-T/G-C content of the microorganism investigated. An example is shown in Table 2.2

Wavelength (nm)	% T<>T/T
280	20.0
254	6.5
235	1.7

Table 2.1: Wavelength dependence of the % T<>T/T ratio in E.coli DNA in the steady state
(Source : Patrick and Rahn, 1976).

DNA	A-T / G-C	% of Total C<>C	% of Total C<>T	% of Total T<>T
H.influencae	1.63	5	24	71
E.coli	1.00	7	34	59
M.luteus	0.43	26	55	19

Table 2.2: Distribution of Pyr<>Pyr in DNA molecules of different base compositions at a fluence of 200 Jm^{-2}, λ = 265nm
(Source: von Sonntag, 1986).

In addition to the dimers, there are other adducts which have a pyrimidine-pyrimidone structure (e.g.Thy(6-4)Pyo) from cytosine and thymine (Fig. 2.5 shows the various photoproducts resulting from UV irradiation). Such

photoproducts are not photoreversible and some open questions remain about the exact structures of the these adducts (Cadet et al., 1985). The pyrimidine adducts, although smaller in yield than the dimer, has been found to contribute greatly to lethality as well as mutations. Other UV-photoproducts include the pyrimidine hydrates which are formed by the addition of water at the 5-6 double bond of pyrimidine bases. They are rather unstable reverting easily upon changes in pH or temperature (Kiefer, 1990). In dry spores, a further adduct, the so-called spore photoproduct is observed. It has not yet been observed in other biological systems (von Sonntag, 1983).

Another product, DNA-protein cross-linking (Sperling and Havron; 1977) should be mentioned because the bacteria, Micrococus radiodurans, which effectively repairs Pyr<>Pyr damage, has its inactivation maximum around 280nm (the maximum of protein absorption), indicating the importance of protein cross-linking in the inactivation of this microorganism (Rahn,1980).

2.5.1 Inactivation and cell killing

It is important to stress that radiation cell killing and inactivation refer to the loss of unlimited reproductive capacity as a result of irradiation. It may not mean the visible disintegration of, or the cessation of all metabolic functions in, the cell. A radiation-killed cell may not exhibit any signs of damage until it attempts to divide and such a "dead" cell may not actually die for weeks or months after exposure to radiation (Coggle, 1983). With bacterial assay systems, lethality (or inactivation) is recognized by failure to form a macroscopically visible colony. Irradiated bacteria can show different kinds of behaviour related to lethality, which can be recognized microscopically; these include the following :

(a) After extensive UV exposure an irradiated cell does not show any cellular growth or division.

(b) A cell continues to grow but fails to form cross walls and to divide. The resulting formation of filaments or other "giant" cell forms is typical of some bacterial strains after low UV irradiation.

(c) UV-irradiated cells continue to grow and undergo a limited number of cell division before these cease.

While investigating the phenomenon of "lethal sectoring", Haefner and Striebeck (1967) distinguished four classes of cells in UV-irradiated E. coli and yeast cells : (1) cells not dividing at all, (2) cells undergoing residual divisions without forming a colony, (3) cells

forming a colony but giving rise to inactive descendants (lethal sectoring), and (4) cells forming a colony without lethal sectoring.

The usual plating techniques (see section 3.2.6) score the sum of classes (1) and (2) as "inactivated" cells and classes (3) and (4) as "survivors". Obviously, the existence of class (3) in the "surviving" population shows that survivors are sometimes not free of UV lesions when they divide.

2.5.2 Biological basis of lethality

The genetic material of a cellular organism has to carry out two important functions in order to reproduce and perpetuate the biological entity : (1) it must replicate itself so that the progeny carry the same genetic specificity possessed by the parent(s), and (2) it must make available, its genetic information. to certain cellular components to provide for all vital phenotypic functions. Consequently, UV photoproducts in the DNA (see figure 2.5) can cause lethality by interfering with either replication, or information transfer, or both.

There is ample evidence, in vitro and in vivo, that UV-induced cyclobutyl pyrimidine dimers interfere with the DNA replication process. DNA polymerase is apparently unable to deal with a dimer and recognize it as two original pyrimidines from which it was formed, and thus terminates synthesis of the daughter strand. There is further evidence that such pyrimidine dimers block the transcription of the genetic information by DNA-dependent RNA polymerase. The

result is incomplete messenger RNA molecules, which the ribosomes translate into truncated polypeptide chains. As should be expected, the blockage at both the replicational and transcriptional level increase with the number of photoproducts, that is, with the UV fluence (Harm, 1980).

2.6 UV Sources

2.6.1 Natural sources

The sun is the largest and most important source of UV radiation on earth. The transmission of solar UV is, however, modified by absorption in the atmosphere. Ozone plays a prominent role in this respect since it reduces, particularly, short wavelength UV. There is practically no solar UV below 300nm at sea level (see fig 2.6). The energy fluence rate of total solar radiation outside the atmosphere amounts to 1393 Wm^{-2}, yielding a total power to the earth of about 10^{17} W. Because of absorption, reflection and scattering in the air, the fluence is much lower at the Earth's surface; in central Europe the annual average lies around $125Wm^{-2}$ (Kiefer, 1990).

2.6.2. Artificial sources

Incandescent matter, at a temperature of 2500K or higher, may emit a significant number of photons with energies inside the UV range. Such incandescent sources emit a smooth spectrum, a continuum, possibly with superimposed lines. In gas discharge tubes, which usually contain mercury at some appropriate pressure, a hot

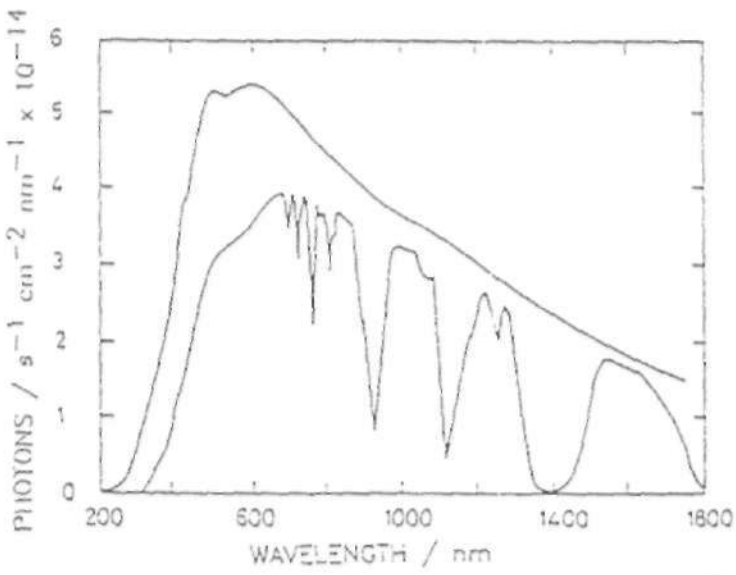


FIGURE 2.6: Photon fluence rate of the sun outside the atmosphere (upper curve) and at sea level with a zenith angle of 60 degrees (Source: Kiefer, 1990)

filament first produces electrons. The voltage across the tube then accelerates these electrons some of which collide with mercury atoms and produce mercury ions. The electrons and positive ions then, by collision, excite other mercury atoms causing them to release energy in the form of fluorescent (UV) radiation. These constitute the two principal methods of producing UV.

Most artificial sources can be grouped into the following categories:

Gas discharges:

- Mercury lamps (low, medium and high pressure)
- Mercury lamps with metal halides
- Noble gas lamps
- Flash tubes
- Hydrogen and deuterium lamps

Incandescent sources:

- Tungsten halogen lamps

Fluorescent lamps:

- Fluorescent tubes
- Fluorescent sun emitters
- Black light UV emitters

Mixed Sources:

- Carbon arc
- Welding arcs

The spectrum of the emitted UV varies from one source to another. In the case of low pressure mercury lamps, a line spectrum is normally emitted. These lines are broadened into bands in high pressure lamps and there may also be a continuum over a wide range of wavelengths. Addition of metal halides increases both the continuum and the number of superimposed lines. In high pressure xenon or hydrogen lamps, there is also a combination of distinct bands with a continuum (Schafer et al.,1977) (fig. 2.7). The arc produced during welding depends on the composition of the electrodes. An example of the emission spectrum produced by a welding process is shown in fig. 2.8.

The spectrum of fluorescent lamps depends on the properties of the fluorescent phosphors (usually, a secret mixture) employed in the envelope. The small UV part of the spectrum of daylight fluorescent lamps stems, however, from the mercury discharge inside the lamp. The amount of emitted UV depends, to some extent, on the glass used in the fluorescent tube. To permit the transmission of UV when the discharge does not take place in free air, gas discharge arcs and other UV sources must be contained within an envelope of quartz or UV-transmitting glass. On the other hand, sources that are designed primarily to emit visible radiation but which also emit significant but unwanted amounts of UV are usually provided with an external screen of glass that does not permit the passage of UV-B and UV-C radiation (Faber, 1982).

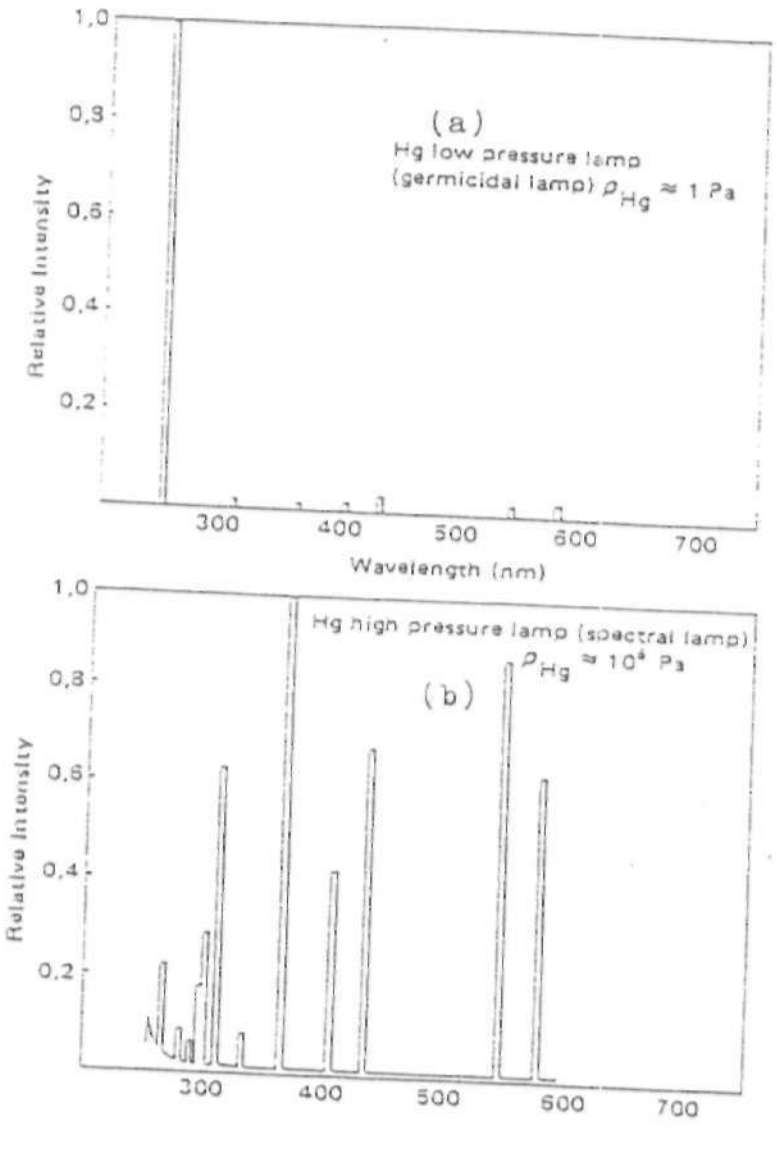


FIGURE 2.7: Emission Spectrum of Mercury lamps
(Source: Faber, 1982)

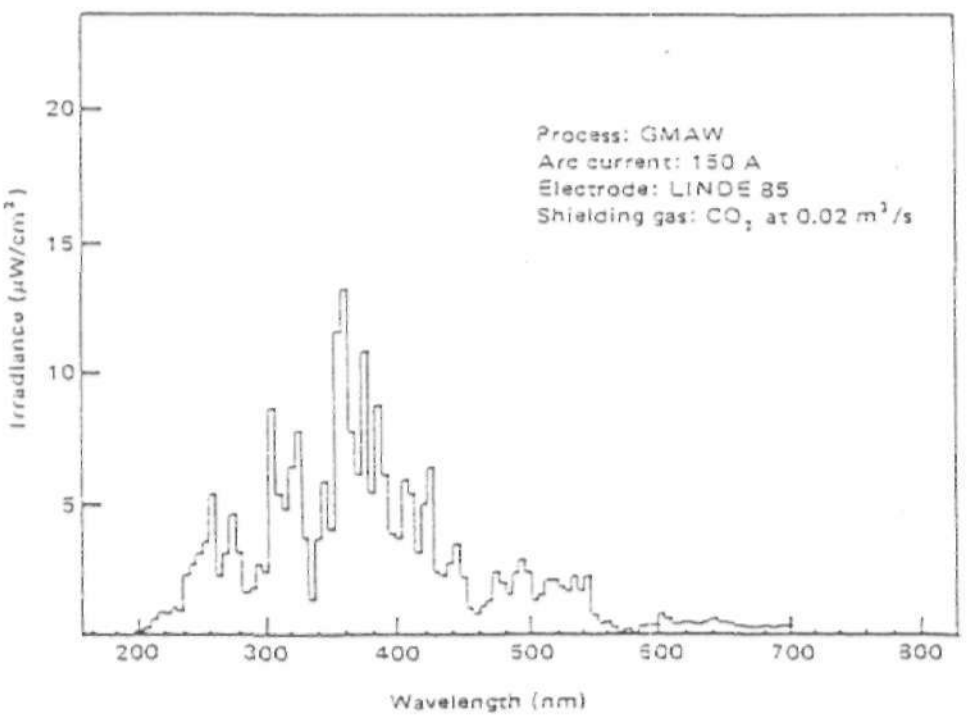


FIGURE 2.8: Emission spectrum from gas tungsten arc welding
(Source: Faber, 1982)

2.7 Inactivation Kinetics

In general, the inactivation of biological material follows two slightly different patterns shown in figure 2.9(a,b,). In fig 2.9(a) the number of viable cells of a species divided by the number of such species originally present (N/No), termed survival, is plotted on a logarithmic scale against the UV-fluence which is proportional to the UV-dose absorbed by the organisms. The degree of inactivation is represented by a straight line over several orders of magnitude of N/No. Such inactivation curves are observed if no repair processes (see section 2.12) occur. In fig 2.9(b) the same quantity is plotted, but at the lower fluence range a shoulder is observed, and only at the higher fluences does the curve become linear. The shoulder is usually due to enzymatic repair processes within the organism. It is clear that only the linear sections of these two curves can be compaired with one another, and the shoulder at the beginning of curve 2.9(b) is therefore often neglected when inactivation data are given. The F_{37} (often called F_0) fluence required to reduce the viable population to 37% ($\bar{e}^1$) is inversely proportional to the slope of the linear part of the curve (in many cases, the shoulder may be quite pronounced and Fo values are inadequate to compare the sensitivity of two organisms) (von Sonntag, 1983).

The exponential survival curve [fig. 2.9(a)] may be described by the equation:

$$N/N_0 = \exp(-F/F_{37}) \qquad (2.5)$$

where N, N_0 and F_{37} are as defined above and,
F is the fluence level for the N/N_0 survival

Using the mathematics of survival curves, the actual target volume can be calculated under the following conditions:

(a) that the inactivation is due solely to irradiation and not to any other biological influences

(b) that the inactivation is the result of direct radiation action in the biological target and not due to the indirect action (diffusion of free radicals into the target volume)

(c) that there is no dose-rate effect which indicates biological repair of some of the targets.

2.8 Bacterial Survival Curves

2.8.1 General description of UV survival curves

Any shouldered curve that becomes exponential within the experimental range of UV fluences can be characterized in terms of : the mean lethal fluence (F_{37}) for the straight line part, the extrapolation number (n), that is, the antilog at which the straight line extrapolation of the exponential portion intercepts the logarithmic ordinate, and the threshold fluence (F_t) defined as the fluence at

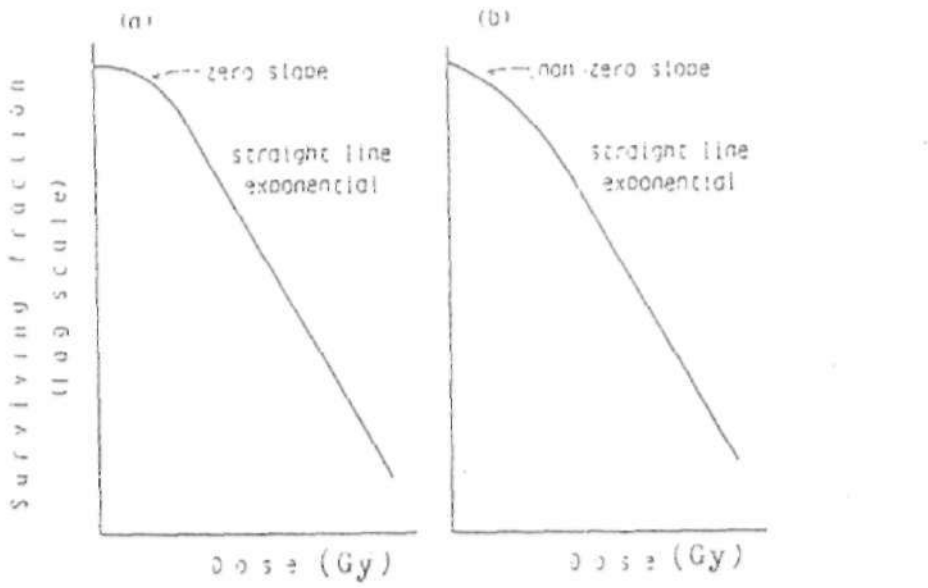


FIGURE 2.9: Typical Curves for the inactivation of biologic organisms
(Source: Coggle, 1983)

(a): Organisms lacking repair mechanism (semi-log plot)
(b): Organisms in which repair processes occur (linear plot)

which the straight line extrapolation of the exponential portion reaches the 100% survival level.

From the definition of F_t and F_{37}, the exponential part of any shouldered survival curve, for a given fluence level (F), can be represented by:

$$N/N_0 = \exp-(F-F_t)/F_{37} \qquad (2.6)$$

The quantity F indicates the approximate magnitude of the fluence with which a biological system can successfully cope (Harm, 1980).

Different bacterial strains have different types of survival curves. Three types are shown in figure 2.10(a,b.c). All three curves are plotted semi-logarithmically, with the surviving fraction on a logarithmic scale against dose on a linear scale. Figure 2.10(a) is a simple exponential curve and indicates repair-free inactivation due either to elevated fluences or the incapacitation of the repair processes in some form.

Figure 2.10(b) is a biphasic survival curve and may, in fact, be the result of irradiating two populations of bacteria, each with different degrees of radiosensitivity (Coggle, 1983). The dotted lines emphasize the dual nature of the curve. Such a biphasic curve may also result from the irradiation of a mixture of rapidly dividing cells which are more sensitive (slope A) and stationary cells which are less sensitive (slope B). Alternatively, the

biphasic curve may be due to some other unknown artefacts (Cerf and Metro, 1977).

Finally, figure 2.10(c) represents a shouldered survival curve, similar to that in figure 2.9(b)

2.8.2 Kinetics of UV absorption

The UV exposure suffered by the microorganism is usually given in fluence units (Jm^{-2}). This unit is related to, though different from, the unit of quantum yield . The quantum yield is the number of molecules formed per photon of radiation absorbed. Since an einstein represents one mole of quanta, the dimension of quantum yield is mole/einstein, the quantum yield can also be defined in terms of a ratio of cross-sections:

$$\Phi = \sigma_p / \sigma_a \qquad (2.7)$$

where σ_p is the conversion cross-section and σ_a, the absorption cross-section.

The conversion cross-section is related to the number of product molecules formed (Δn_p) when n_s molecules of the parent are exposed to a small photon fluence F, by:

$$\Delta n_p = \sigma_p n_s F \qquad (2.8)$$

This expression is valid under conditions where there is negligible absorption by the substrate within the illuminated medium and this holds true for most photobiological systems.

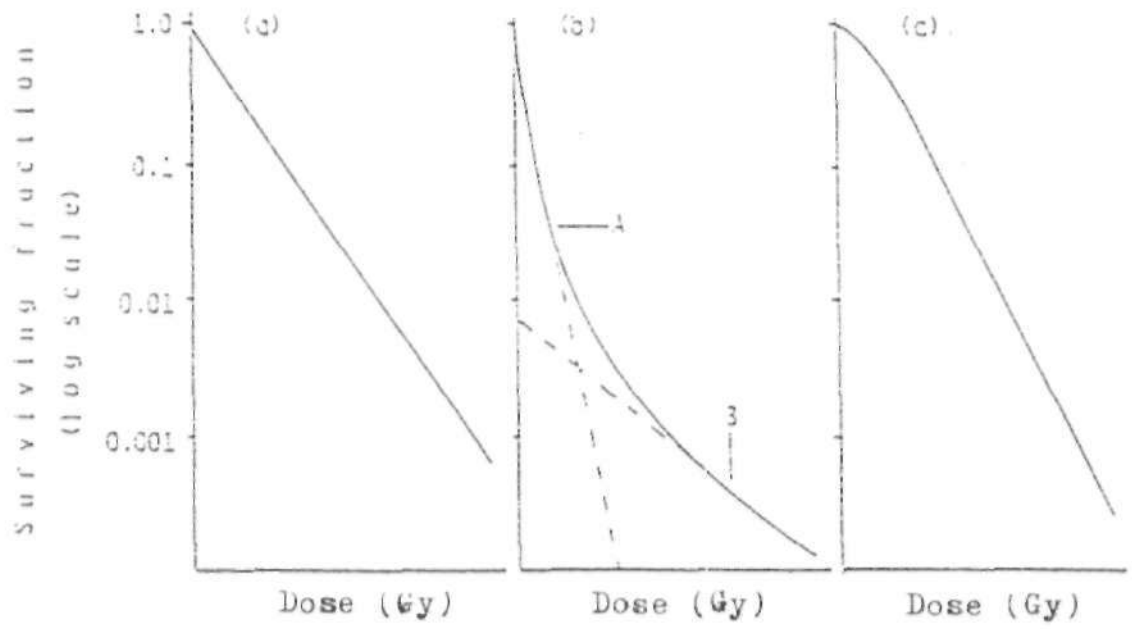


FIGURE 2.10: Bacterial Survival curves
(Source: Coggle, 1983)

(a) Single-heat exponential survival curve
(b) Biphasic survival curve
(c) Shouldered survival curve

If several products are formed along different reaction channels $S \rightarrow \alpha_1 P$, $\alpha_2 P$,..., (α_i = stoichiometric factors) then,

$$n_S = (n_S)_o \exp.-(\Sigma\sigma_1/\alpha_i F) \qquad (2.9)$$

where $(n_S)_o$ is the number of parent molecules at the start of the illumination, and F the intergral fluence.
In the special case where none of the products absorb light at the photolysis wavelength,

$$n_S/(n_S)_o = A/A_o \qquad (2.10)$$

A = absorptivity for the irradiation level

A_o= absorptivity for zero fluence

The equations (2.9) and (2.10) permit the determination of $\Sigma\sigma i/\alpha i$

For 254nm quanta the F_{37} (defined in section 2.8.2) is given by:

$$F_{37} = \frac{4.71 \times 10^5}{(254) \times 0.23 \times (254)} \ [Jm^{-2}] \qquad (2.11)$$

where (254) is the extinction coefficient at 254nm (von Sonntag,1983)

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found that, in practice, bacterial death rate is not logarithmic (Stumbo, 1965).

Most of the survival curves showing a deviation from logarithmic order give either convex or concave curvature on a semilog plot. These curvilinear survival curves are commonly attributed to experimental artifacts, such as mixed population of test organisms, flocculation and deflocculation of cells during the heating period (Stumbo, 1965), heat activation for spore germination and aggregation of cells (Hiatt, 1964). However, experiments which are done under conditions which eliminate these so-called artifacts still produce curvilinear survival curves. Han (1975) reported concave survival curves for the thermal inactivation of spores of the bacteria, Bacillus cereus.

The cause of non-linear survival curves has also been explained by the multiple critical sites theory (Meynell and Meynell, 1970) and heterogeneity of spore resistance (Han, 1975). In the present context, however, since mainly dry spores were used in the heat treatment, the artifacts referred to above, do not apply. So only the the multiple critical sites and heterogeneity theories need to be considered.

2.9.2 Multiple Critical Sites theory

According to the model used, the probability that a cell survives is equal to the probability that it has less than some fraction of N critical sites inactivated. The following first order equation is assumed:

2.9 Effect of heat on the bacterial spore

It is known that bacterial spores are inactivated when subjected to heating at ultra high temperatures (above 200 (Murrel, 1969). Heat injury of spores has been variously attributed to: denaturation of vital spore enzymes; damage to the membrane resulting in leaking of cytoplasmic constituents as well as increased sensitivity to inhibitory agents, such as sodium chloride, and finally, structural damage seen in the electron microscope as overt blistering and disruption of the intergrity of the spore membrane (Grecz and Bruszer, 1980).

Damage to the spore chromosome has also been inferred from the occurence of heritable mutations induced either by dry or wet heat (Zamenhof, 1960; Kadota et al., 1978). It was postulated that heat modifies specific sites on the spore chromosome. So far, however, in many laboratories working on this field, no strand breaks have been detected in spores although in vegetative cells, heat injury invariably induces single and double strand (DNA) breaks. Furthermore, the cell's own apurinic endonuclease is assumed to play some part in the heat induced DNA strand breakage in vegetative cells (Grecz and Bhatarakamol, 1977).

2.9.1 Kinetics of Bacterial Heat Inactivation

The death rate of bacteria and their spores subjected to lethal heating is generally considered to be logarithmic (Stumbo, 1965). However, many researchers in the field have

$$X = N[1-\exp(-kt)] \qquad (2.12)$$

where t = the time

N = initial number of critical sites

k = the rate (assumed constant) of inactivation of individual sites

X = the number of critical sites inactivated at time, t.

"Critical sites", in this context, is considered from the molecular level and could be a molecule or molecular group susceptible to thermal damage.

Equation 2.10 is based on the following assumptions :

(i) Inactivation of individual sites follow first order kinetics equation and occurs at random

(ii) All sites are identical and of equal heat resistance

(iii) The bacterial population is homogeeous with regard to heat resistance(Moats, 1970)

2.9.3 Innate Heterogeneity theory

Here two situations are distinguished, namely, innate heterogeneity of spore population and heat adaptation during a heating period.

Under the innate heterogeneity theory, the given population is assumed to be heterogeneous with regard to heat resistance. The distribution of bacterial heat resistance is assumed to be binomial and to reduce to the normal (Gaussian) distribution as the number of samples

become large. Defining a "rate of destruction", K, as the probability of destruction of a spore per unit time (K is assumed constant for innate heterogeneity), the following "survival" equation is arrived at (Han et al,1975):

$$\text{Log } C(t)/C(0) = -K_o t + (\sigma/2)t^2 \qquad (2.13)$$

Where C(0) is the initial concentration of spores

C(t) is the concentration of spores at time t

K_o is the most probable value of K among the spores

σ is the standard deviation

Equation (2.11) represents a parabolic curve. Therefore, based on this model, it is concluded that the innate heterogeneity of spore population produces parabolic survival curves on semi-log paper during at least the initial 20 decimal reduction time.

Under the heat adaptation theory it is assumed that:
(i) spores develop heat resistance when exposed to heat
(ii) there exists a maximum level of resistance attainable by each spore before it dies
(iii) it takes some finite time to acquire resistance.
(iv) The rate of destruction is defined to be a function of heating time

With these assumptions, a "survival" equation is obtained which results in an exponential curve on semi-log paper.

2.10 Mechanism of action of heat and radiation

A number of investigators since the early 1950's have reported that relatively mild doses of radiation sensitized bacterial spores , and many other microorganisms as well as viruses, very significantly to subsequent heat injury. In 1965, Grecz reported a 13-fold sensitization of the resistant spores of C. sporogenes as well as an 8-fold sensitization of B. subtilis spores (Grecz, 1965). These studies, which were carried out with ionizing radiation, were further confirmed by similar results on spores of food-related organisms including C. perfringens and C. botulinum and have engendered much interest in combination processing using radiation and heat to eliminate microorganisms from food products.

The mechanism of synergistic effect of radiation and heat is not yet clear, but may perhaps be traced to the basic biological modes of action of these two forms of energy. Heat intensifies the vibrational and rotational motion of the molecules. This leads to configurational changes causing activation of enzyme-substrate complexes or eventual denaturation of enzymes and nucleic acids. Heat levels of concern in microbiology, however, generate relatively weak forces which influence the secondary and tertiary folding of biopolymers but generaly do not disturb the atomic structure. In the light of this, it has been proposed that synergism may be due to the fact that lethal heat inactivates repair enzymes, while radiation sufficient to injure the spores leaves these enzymes virtually

unharmed (Grecz et al. 1985).

2.11 Repair of UV-induced damage

Photoreactivation, a repair mechanism in which UV-induced pyrimidine dimers are monomerized by an enzyme in the presence of near UV or short-wavelength visible light (see equation 2.14), indicates that dimer formation is the major cause of UV-induced damage.

$$PRE + Pyr<>Pyr \underset{\longleftarrow}{\longrightarrow} PRE \ldots Pyr<>Pyr \longrightarrow PRE + 2Pyr \quad (2.14)$$

The fact being that post-irradiation exposure of colls to near UV or short wavelength visible light results in 50 - 90% enhancement in the survival (von Sonntag, 1986). In connection with dimer damage, it should be mentioned that many dimers have to be formed if the effect is to result in inactivation or loss of colony forming ability; it is not enough to induce merely one such lession in the microorganism. Even in a simple system such as the simian virus SV 40, as many as 40 dimers are required to prevent its propagation (Brown and Ceratti, 1986).

Function	Proportion of total lethality (%)
DNA replication	45
RNA synthesis	30
Regulation of gene transcription	15
Termination of DNA replication	15

Table 2.3: Lethal effect of DNA damage (of the virus SV40) attributable to the disruption of individual genetic functions by 254nm UV -irradiation

Source: (Brown and Ceratti, 1986)

Other modes of UV-damage repair include :

(i) Replacement of UV-damaged nucleotides; for example, in excision - resynthesis repair, a UV photoproduct, together with a sequence of adjacent nucleotides, is eliminated from the DNA molecule and the correct sequence is synthesized .

(ii) Combination of undamaged regions in replicating DNA molecules (an example of which is post-replication repair), in which undamaged DNA regions are replicated and combined in such a manner that an intact DNA molecule, identical with the original is formed (Harm, 1980).

In addition to these repair mechanisms, it also known that UV-damage triggers an SOS response which in bacteria, causes increased repair activity (some of which may in fact be error-prone) as well as inhibiting cell division.

CHAPTER THREE

Materials and Methods

The investigations were carried out on spores of the wild strain of the bacteria, Bacillus subtilis obtained from the Institute of Space Medicine, Cologne, F. R. G.

Bacillus species, in general, start with the formation of endospores, when at the end of their growth phase, the easily accessible carbon, nitrogen and phosphate sources are used up. For good growth and sporulation, the medium has to contain easily metabolizable carbon and nitrogen sources, phosphate, magnesium, iron and zinc, as well as such substances which are needed for sporulation (manganese and calcium). Another possible way to achieve sporulation is to transfer a fast growing culture from a rich medium to a poor one (Horneck, 1989).

3.1 Stages in Sporulation

Sporulation passes through a number of phases until resistance to radiation and heat is attained. Spore formation starts with ingrowth of the cytoplasmic membrane of the parent cell at one end. The protoplast which is partitioned off contains part of the nuclear material, that is, a genome. It is characteristic of spores undergoing formation that no cell wall is formed as occurs in vegetative cell division; instead, the cytoplasmic membrane of the parent cell grows around the spore protoplast. This

phase is over when a completely seperate cell which is surrounded by two cytoplasmic membranes and is still osmotically fragile (the forespore) has formed. Incorporation of peptidoglycan layers between the membranes and of the forespore forms the cortex . This is the commencement of the refractility of the spores. There is active Ca^{++} uptake from the surrounding medium via the parent cell . The uptake of Ca^{++} is paralled by the synthesis of dipicolinic acid (DPA), which forms chelate complexes with the calcium (Fitzjames and Young, 1969). Vinter (1961) found that synthesis of DPA is accompanied by an increase in the resistance to heat. Two hours before this, an increase in resistance to radiation accompanies the development and incorporation of cysteine rich proteins into the spore.

In the final phase of sporulation, the layers of the coat becomes more dense, and the water content of spore decreases to a minimum of 15 per cent. The refractility of the spore is a maximum at this stage; that is, it is detectable by phase contrast.

3.2 Cultivation of the Spores

3.2.1 Sporulation Medium

The sporulation medium used was constituted as follows : 3.2g Difco Nutrient Broth, 1g Potassium chloride and 200ml distilled water. The mixture was autoclaved in a 1-litre Fernbach flask, after which the folowing salts were

a - parent cell undergoing lysis

b - exosporium

c - coat membrane

d - outer sore membrane

e - cortex

f - later cell wall after germination

g - inner spore membrane

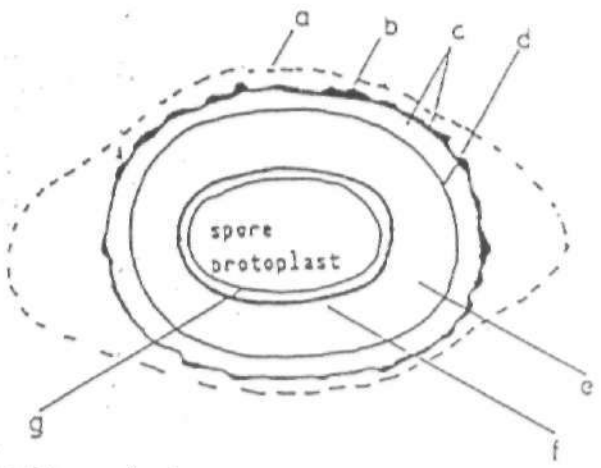


Figure 3.1(a): Diagrammatic section through a bacterial spore

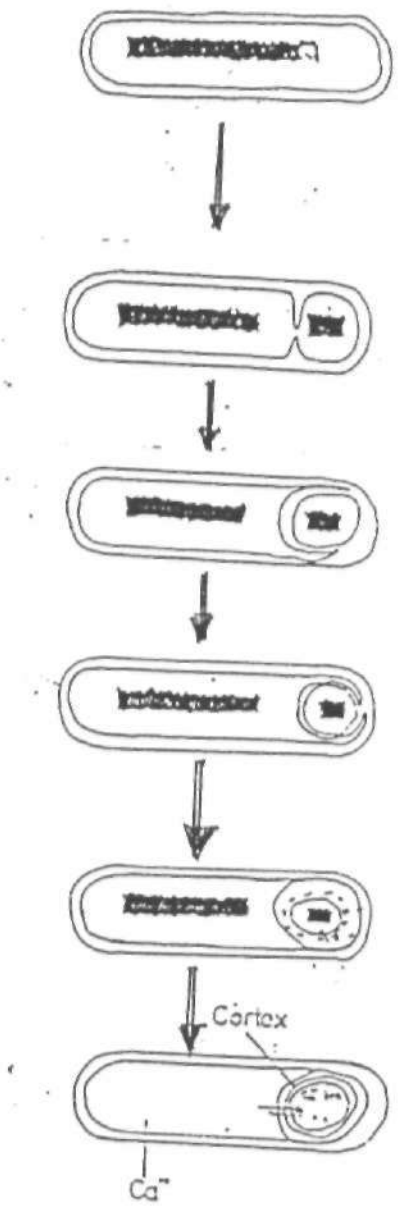


Figure 3.1 (b): Stages in the development of a bacterial spore

added, 0.4ml of 1M calcium chloride,0.2ml of 0.01M Ferric chloride, 0.04ml of 0.1M Manganese sulphate, 0.02ml of 0.1M Magnesium sulphate and 0.4ml of 50% glucose. Each of these salt solutions was seperately autoclaved before being added to the medium.

3.2.2 Growing and Harvesting of the Spores

The parent spores were first incubated for about 10 hours in a complete medium made up of : 8g DIFCO Nutrient brot, 4g Sodium chloride, 1 litre distilled water and solidified with 16g Difco Bacto Agar. From this, two innoculating "loop-fulls" were suspended in 5ml Nutrient Broth solution. After thorough mixing, 0.5ml of this suspension was put in the sporulation medium and incubated for 64 hours at 37^{o}C with shaking (approximately 150rpm). The spore fraction was determined with a counting chamber. The culture was found to contain 80-90% spores. The spores were harvested by centrifugation at 7000rpm for 10 minutes and washed thrice with double distilled water.

3.2.3 Cleaning of the spores

The sediment of spores was re-suspended in a solution made up of : 7ml of 0.01M tris buffer (pH 7.2) and 0.01ml of 0.002g/ml DNAse, 0.1ml of 0.01M Magnesium sulphate and 0.1ml of 0.02g/ml Lyzozyme. After incubation for 30 minutes in a water bath at 37^{o}C, the sample was heated to 80^{o}C in a water bath for 10 minutes and then immediately centrifugated (7000rpm) for 20 minutes. The sediment was then whirled and washed four times in double distilled

water after which the purity of the spores was examined microscopically and the titre of germ determined.

3.2.4 Density Gradient Centrifugation

To further purify the spores and seperate the pure spores from the spore fragments and vegetative cell remnants, it was necessary to do the density gradient centrifugation; this was done according to the method described by Tamir and Gilvarg (1966).

A solution containing 76% urografin was diluted to 70% and 50% urografin respectively. Then, with the help of a gradient mixer, 12ml of a continual density gradient (70%-50%) of urografin was produced; 0.5ml of the spore solution (containing 10^9 - 10^{10} cells/ml) was put on the gradient. Centrifugation (at 16,000 rpm) was done in the SW40 rotor at 20 ^{o}C for one hour without break. The spores, as well as cell fragments, were accumulated in three bands; the middle band containing the pure spore fraction was removed with a gradient unloader. This was then washed four times with double distilled water in order to clean the spores of urografin.

3.2.5 Determination of the Total Cell Count by Microscopy

It is necessary to know the number of bacterial cells per unit volume before exposure to radiation in order to carry out quantitative evaluation.

The suspensions which were used contained about $_{10}{}^{10}$ to 10^{11} spores per mililitre. The exact total cell count was

determined with a haematocytometer (Thoma chamber). The counting chamber is a thick, flat-ground slide in the centre of which there is a strip bounded by two ground channels. A square network comprising 400 small squares of edge 0.05mm is etched into the strip; 16 such small squares making a large square of area 0.04 mm . The surface of the strip is 0.02 below that of the slide so that a chamber is formed when a flat-ground cover glass (0.2mm thick) is placed on top. The edge of the cover glass is placed firmly on to the the slide so that Newton's rings are visible from the edges. This ensures that, when the filled innoculation loop is applied, only one cell layer is drawn by cappilary forces underneath the cover glass (Drews, 1968). The ideal number of cells for the counting chamber is about 10^7 per milimetre. For this reason, a 1 : 1000 dilution with double distilled water is done before introduction into the counting chamber. After this, a sterile innoculation loop was used to take one drop of the 10^{-3} dilution and introduce into the counting chamber.

The evaluation under the microscope was carried with a 40 x magnification with phase contrast. The spores were counted diagonally over four small squares. The results for several small squares were averaged and multiplied by the chamber factor (2.10^7) in order to obtain the concentration of the 10^{-3} dilution.

Mean count x chamber factor = titre/ml 3.1

This method includes cells which may have been damaged or are already dead. However, the interpretation of irradiation results is based on viable cells which are damaged or killed by exposure to radiation. Hence the titre thus determined is merely a starting point for the determination of the live cell count.

3.2.6 Determination of the live cell count by serial dilution and plating out

A completely viable cell is recognized as such by its unlimited division and growth in nutrient media, to form a colony which is detectable on inspection. To determine the organism count in a dense suspension of bacteria, it was necessary to dilute the latter since, otherwise, on plating a sample on Petri dishes, the colonies form a confluent bacterial lawn. Since the number of live organisms per litre is unknown, a dilution series was prepared and a sample of each dilution plated out and incubated at 37^{o}C. The dilutions were selected so that they result in between 50 and 150 colonies per plate, which is favourable for counting and the subsequent evaluation. When there are fewer than 50 colonies per plate, the small number of samples has a large effect on the determination of the titre. More than 150 colonies per plate falsifies the count because of the poor resolution resulting from overgrowth of indidual colonies (Drews, 1968).

The dilutions were produced using Oxford, Eppendorf and glass pipettes. The procedure used was such that, for example, a 20μl Oxford pipette was used to remove 0.02ml

from the irradiation vessel (10^0 dilution) and place in the tube for the 2x10 dilution. After thorough mixing, 0.1ml was removed from this tube and placed in the tube prepared for the $2x10^{-3}$ dilution. The following point had to be taken note of : for each dilution, a new sterile tip or, in the case of glass pipettes, a fresh sterile pipette was used. After whirling, 0.1ml of the bacterial suspension was removed from the tubes containing the relevant dilutions and plated. A spatula was used to distribute the innoculum uniformly over the surface of the agar. The mean count for the samples was used for calculation of the titre.

3.3. Statistical Analysis and Estimation of Errors

In order to compensate for the random deviations from the mean for a sample, several samples were plated out. After the innoculated agar plates had been incubated, the colonies were counted with the naked eye and the number of organisms in the initial suspension (10^0 dilution) was calculated. The titre was calculated by the following formula:

$$T = \frac{\text{total number of colonies counted}}{P \times \text{dilution} \times 10^{-1}} \qquad (3.2.)$$

P = number of parallel plates

10^{-1} results from 0.1 ml being plated out.

The standard deviation from the mean was calculated as follows :

$$\sigma = \sqrt{\{(x-\bar{x})^2/(n-1)\}} \qquad (3.3.)$$

x = number of colonies on a parallel plate

$\bar{x}$ = mean number of colonies per plate

On irradiation of several samples with the same fluence, the error is reduced by $1/\sqrt{L}$, L being the number of parallel irradiations :

$$S_{\bar{x}} = \sigma / \sqrt{L} \qquad (3.4)$$

is the standard error of the arithmetic mean (Sachs, 1973)

3.4 Experimental Procedure

3.4.1 Preparation of samples of bacteria for irradiation in the dry system.

To prepare the irradiation samples, 40 l of a diluted stock suspension, was pipetted dropwise on to sterile polyethylene (25 x 25mm) surfaces. This was allowed to dry overnight to reach equilibrium between the cells and the surroundings. Investigations at the Institute of Space Medicine, Cologne, F.R.G. had shown no change in the spores after longer drying times (Woizenko, 1986).

For each experiment, 20 polyethylene surfaces were coated for the irradiation at room temperature and under atmospheric pressure.

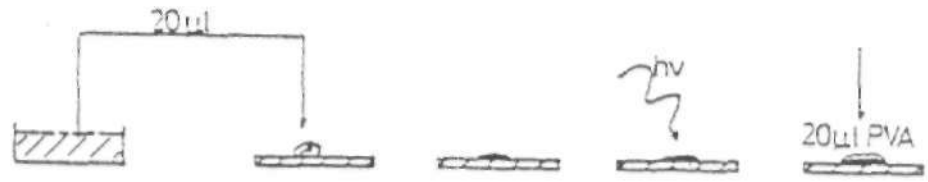
3.4.2 Irradiation in suspension

Five mililitres of spore suspension was irradiated, while stirring slowly, in 7cm glass Petri dishes. Defined quantities (20fl) were removed from the irradiation vessel at set time intervals (0, 15, 30, 60, 100 and 140 seconds), and were diluted in 1ml of distilled water. The control was in the form of a sample which had not been irradiated. After appropriate dilutions with Eppendorf and/or glass pipettes, the irradiated samples were plated out in nutrient agar (8g of DIFCO Nutrient Broth, 4g of Sodium Chloride, 1 litre double distilled water and 16g DIFCO Bacto Agar).

3.4.3 Irradiation of air-dried samples

The coated polyethylene samples were placed in 7cm Petri dishes for the irradiation . For constant UV fluence, the samples were irradiated for various time intervals. A similar procedure was adopted for the heat treatment except that in this case the samples were heated in a thermostat-equipped oven instead of, or before, being irradiated.

After the irradiation and/or heat treatment, it was necessary, for the evaluation, to detach the treated layer of spores completely from the polyethylene surfaces. A water-soluble synthetic material, polyvinyl alcohol (PVA)

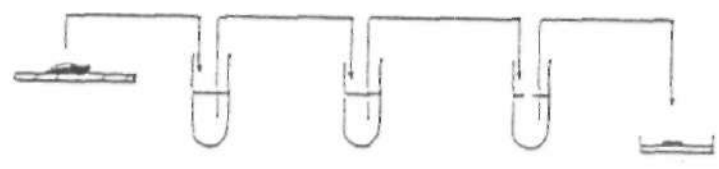


(a)

Spore suspension — Cover glass with drops — Dry spore — Irradiation — Coated with PVA

3 .10^7/ml — 6 .10^5/ml — sample

removal



(b)

FIGURE 3.2: Technique for colony forming assay

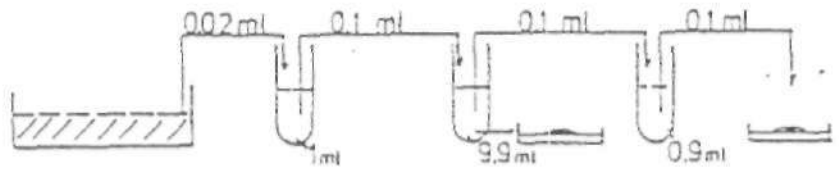


FIGURE 3.3: Dilution series for 0 control

from KALLE AG, WIESBADEN, FRG, was used for this purpose, a 10% solution being added dropwise to the dried spores and forming, after two hours, a transparent film to which the spore layer adhered. Careful removal of the PVA film allowed complete re-suspension of the dried spores in distilled water and thus determination of the ability of the spores to the divide. (Fig. 3.2 surmarizes the technique for the determination of the survivability of spores).

Each coated polyethylene sample produced one inactivation result. For each irradiation event, the Petri dish was rotated clockwise by 180^{0} in order to compensate for slight inhomogeneities in the irradiation field.

All experiments were performed three times. Survival fractions were calculated as number of colonies after irradiation per number of colonies without exposure.

3.5 Bacterial Strain and irradiation conditions

The Bacillus strain used for these experiments was the wild-type 168. The irradiation source was a low pressure mercury lamp, model HNS12 produced by OSRAM, Federal Republic of Germany. The lamp output has 85% UV of wavelength 253.7nm and smaller peaks at 365nm and 465nm. So, for experimental purposes, it is assumed to be a 254nm wavelength UV source.

All procedures were carried out under fluorescent yellow light (PHILIPS, 30W gold). The aluminium-coated polyethylene surfaces used in the experiments were supplied by MERZ VERPAKUNGSMASCHINEN GMBH., Lich, FRG.

3.5.1 Potassium Ferrioxalate Actinometry

Actinometry was used to determine the UV fluences employed in the irradiations. The method used is as described by Hatchard et al. (1956). The procedure involves preparing the actinometry and calibration solutions and then using them to determine the UV fluences, as performed by Schenk (1989).

The actinometry solution was prepared by dissolving 2.947g of 0.006 M potaassium ferrioxalate [$K_3Fe(C_2O_4)_3 .3H_2O$] crystals in 800 ml water, 100 ml of 1N H_2SO_4 and bringing to litre with water.

Calibration solution (0.4×10^{-6} mole ml^{-1} of Fe +++) was prepared (using volumetric flask) by diluting one part of 0.1 M FeSo4 with 250 parts of 0.1 N H_2So_4 .

Phenanthroline and buffer solutions were prepared by, respectively, dissolving 1g of 1.1 phenanthroline monohydrate in 1 litre water and mixing 1 N sodium acetate with 360 ml of 1N H_2So_4 and bringing to one litre with water.

Calibration was done by placing in a series of 20-ml calibrated vessels (stoppered), 0, 0.5, 1.0 ... ,4.5, 5.0 ml calibration solution. These were then topped to 10 ml with 0.1N H_2So_4 , then 2 ml phenanthroline solution was added and, after mixing, the mixture was topped to 20 ml with the buffer solution. This was allowed to stand for 30 minutes (final pH was 3.5), then the optical density was measured at 510nm in a 1-cm cell, using as a blank the reaction mixture containing no calibration solution. A

graph of optical density versus amount of Fe+++ added was plotted (fig 3.4) and this provided a factor for conversion of optical density to amount of ferrous ion.

The actinometry procedure was identical to that for calibration except that the sample was irradiated actinometer solution instead of calibration solution. The samples consisted of 5 ml of actinometry solution irradiated with different UV fluences. After topping to 20 ml as described in the calibration procedure, the optical density was measured using as a blank, reaction mixture containing unirradiated actinometer solution. The conversion factor was applied and this gave the amount of ferrous ions formed by the irradiation. This was then converted to einsteins (the radiation fluence) by dividing by the quantum yield ,which is 1.26 for 254nm (Jagger, 1976).

The calculations are as follows:

$B = ck/t$ $(Jm^{-2}\ s = Wm^{-2})$, where

t = irradiation time in s

c = concentration of Fe+++ obtained from gragh in mol/l

$$k = \frac{V.v.E}{f.Q}$$

where, V = volume of the irradiated solution, v = dilution factor, E = energy in J/Einstein, Q = number of particles per photons absorbed, f = area of petri dish used for the experiment, $B = 1.81\ Jm^{-2}$ for lamp-petri distance of 30cm and f = surface area of petri dish.

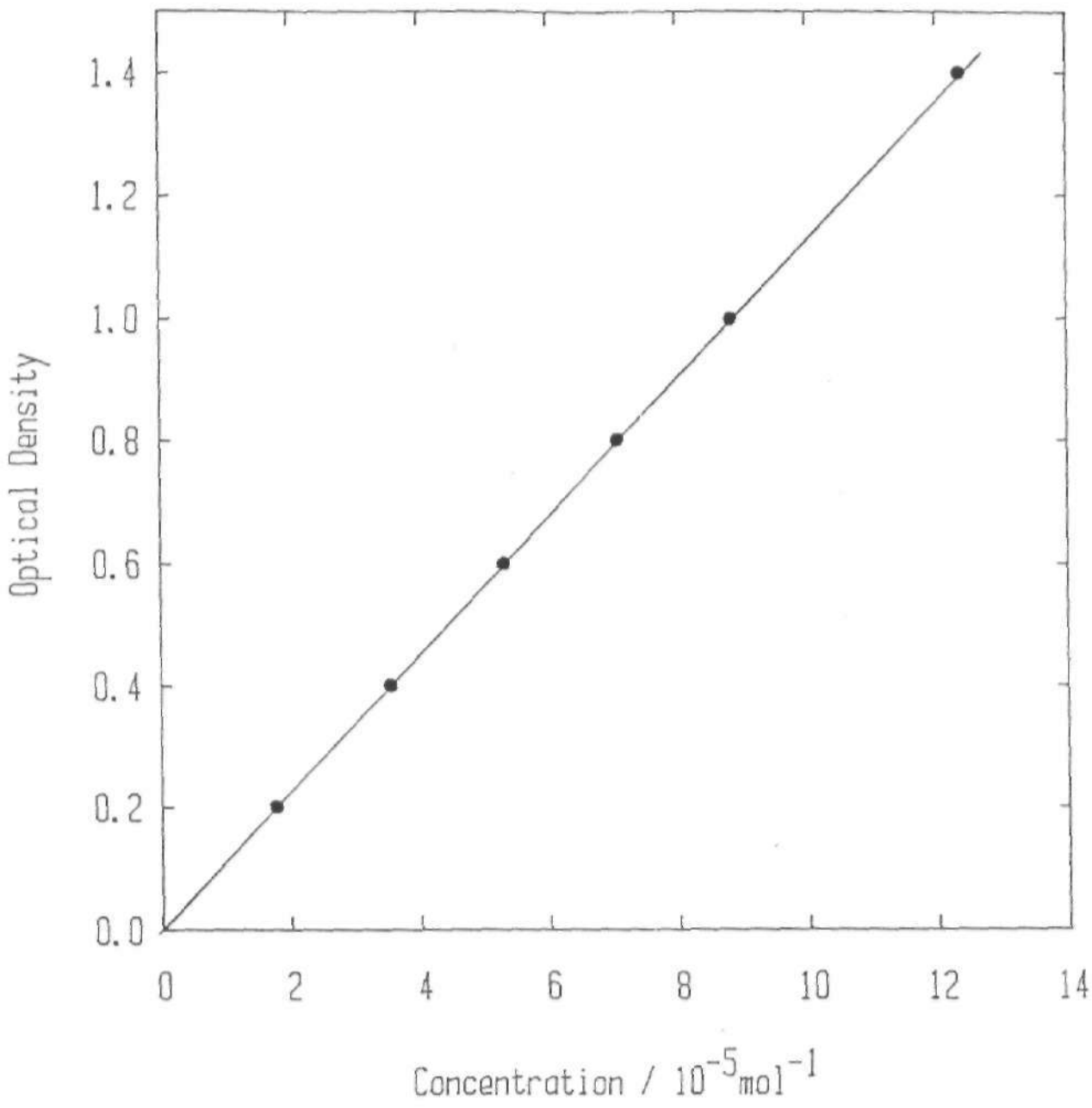


FIGURE 3.4: Calibration curve used for actinometry

3.6 Nature of the experiments

3.6.1 Survival Curve of Spores in suspension

To see the nature of the survival curve for suspension-irradiated spores so as to compare with irradiation in the dry state, the spores were irradiated while suspended in double distilled water. Different spore concentrations were used in order to observe the effect of spore concentration on inactivation.

3.6.2 Irradiation of spores on Polyethylene

To examine the effect of the polyethylene surface on the spores and also compare the wet irradiation with irradiation in the dry state, both wet and dry spores were placed on sterile samples of polyethylene and irradiated. The same spore concentration was used in both cases so as to eliminate any shielding effect.

3.6.3 Effect of Heat

To study the effect of heat, spore samples on polyethylene surfaces were subjected to various degrees of heat treatment after which they were irradiated with 108.5Jm UV fluence.

3.6.4 Fluence-dependence of Synergism

To have an idea of idea of the fluence-dependence of synergism between UV radiation and heat on the spores, a second experiment similar to the above was carried out with higher fluence being used.

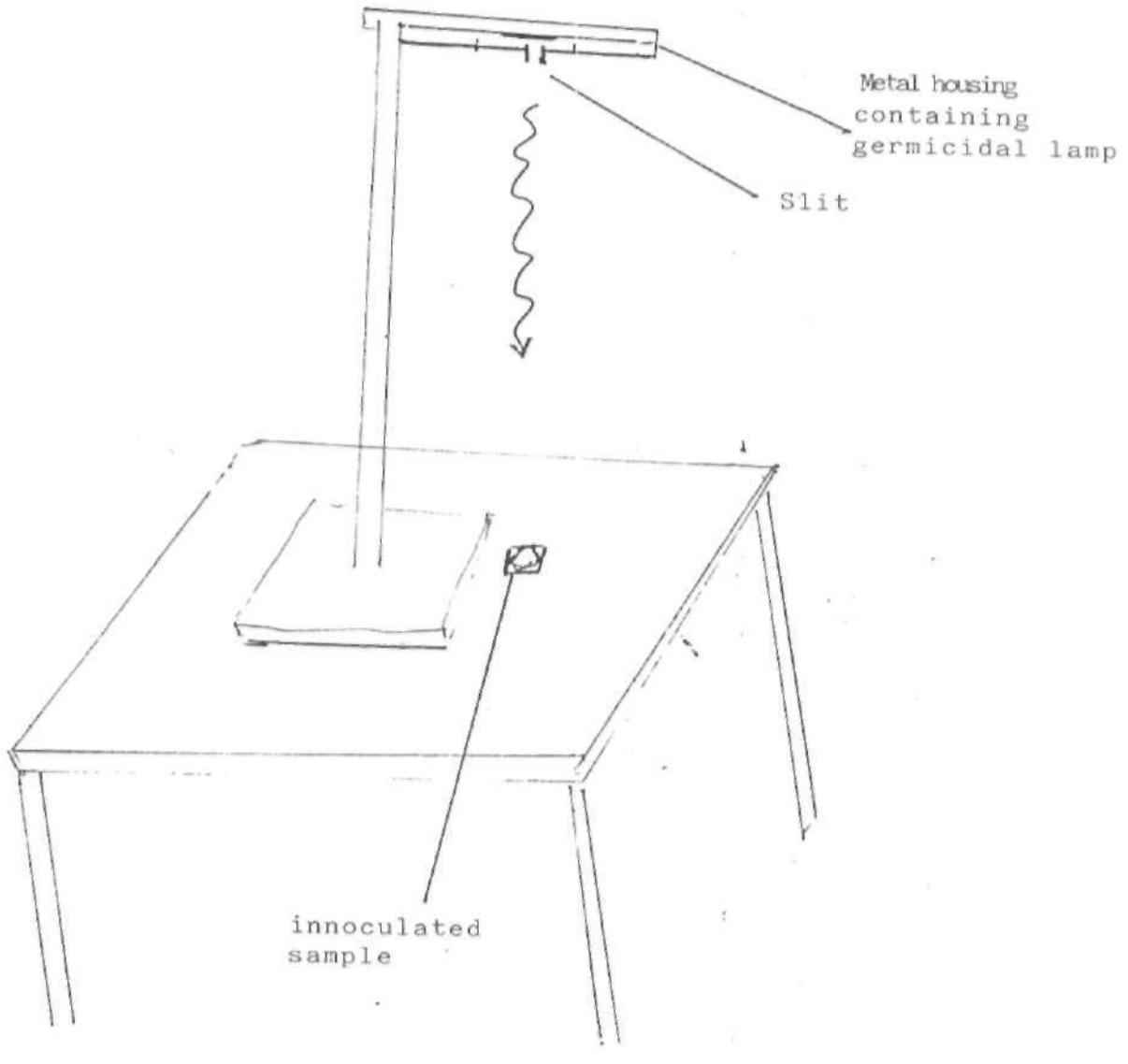


Figure 3.2(a): Experimental set-up

3.6.5 Effect of Prolonged Heating

Dry spores of polyethylene were heated for 30 minutes at 50 C before being irradiated and their survival was compared with that of spores exposed to UV irradiation only.

Previous experiments, which indicated an enhanced inactivation rate for spores heated to temperatures above 90 C provided the impetus for further examination of inactivation of spores subjected to prolonged heating at 100 C. Here, two parallel inactivation experiments were carried out: the first with only heated spores and the second with with spores subjected to both heating and mild UV irradiation ($54Jm^{-2}$).

Finally, the shape of the survival curve ofspores subjected to elevated UV fluences was examined.

3.6.6 Plating Efficiency

In microbial as well as mammalian assay systems, the number of cells recovered after plating and incubation often assumes a high degree of importance as it indicates how efficient the recovery technique is and/or the percentage of viable cells in the initial population . This is termed "plating efficiency". It is known that mammalian cells usually have a much lower plating efficiency, compared with bacteria, since they are usually plated outside their natural habitat. With bacteria, the method of recovery is often the key element influencing plating efficiency, assuming that all the plated clls are viable.

Plating efficiency is defined by:

$P = N / N$ where,

N is the number of cells plated and N is the number recovered.

In connection with this study, preliminary experiments were done to find out which method of recovery yields the highest plating efficiency. The method of coating the treated polyethylene samples with polyvinyl alcohol before agitating in double distilled water was compared with simply agitating the treated samples in an electrically powered shaker.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1(a) Adhesion of B. Subtilis spores to polyethylene

Electron microscopic examination of the dried spores on polyethylene was made in order to have an idea of the degree of spore adhesion to polyethylene.

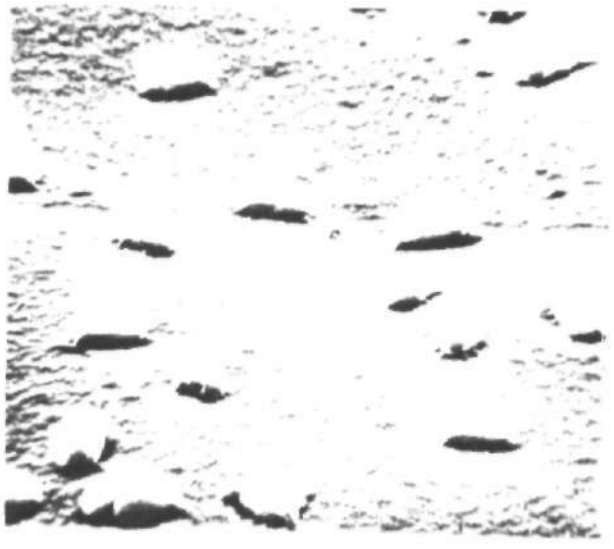
Figures 4.1(a) and 4.1(b) are, respectively, 8.5×10^3 and 1.7×10^4 magnifications with the filters at 75 \degrees relative to the horizontal, while figure 4.1(c) is an 8.5×10^4 magnification with the filter at 70 degrees to the horizontal. Keeping the filter at 75 degrees to the horizontal enables the observation of certain ultra structures with which the spores adhere to the surface. These results were further confirmed by determining the plating efficiency of the spore population.

4.1(b) Plating efficiency

The results of the plating efficiency experiments are as follows:

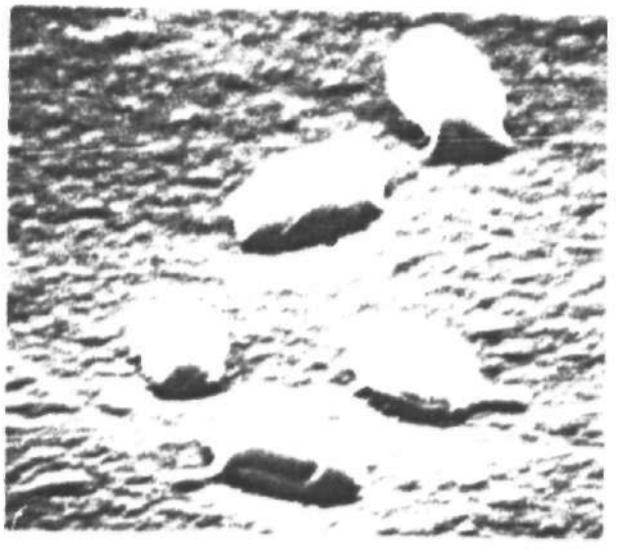
Method of recovery	Plating efficiency (%)
shaking in distilled water	87 + 1.2
coating with PVA before shaking	93 + 2.0

Coating with PVA before shaking in double distilled water yields a higher plating efficiency. The fact that the



(a) Magnification: 8500

Angle: 75 degrees



(b) Magnification: 17000

Angle: 75 degrees



(c) Magnification: 8500

Angle: 70 degrees

FIGURE4.1: Bacilus subtilis Spores on Polyethylene

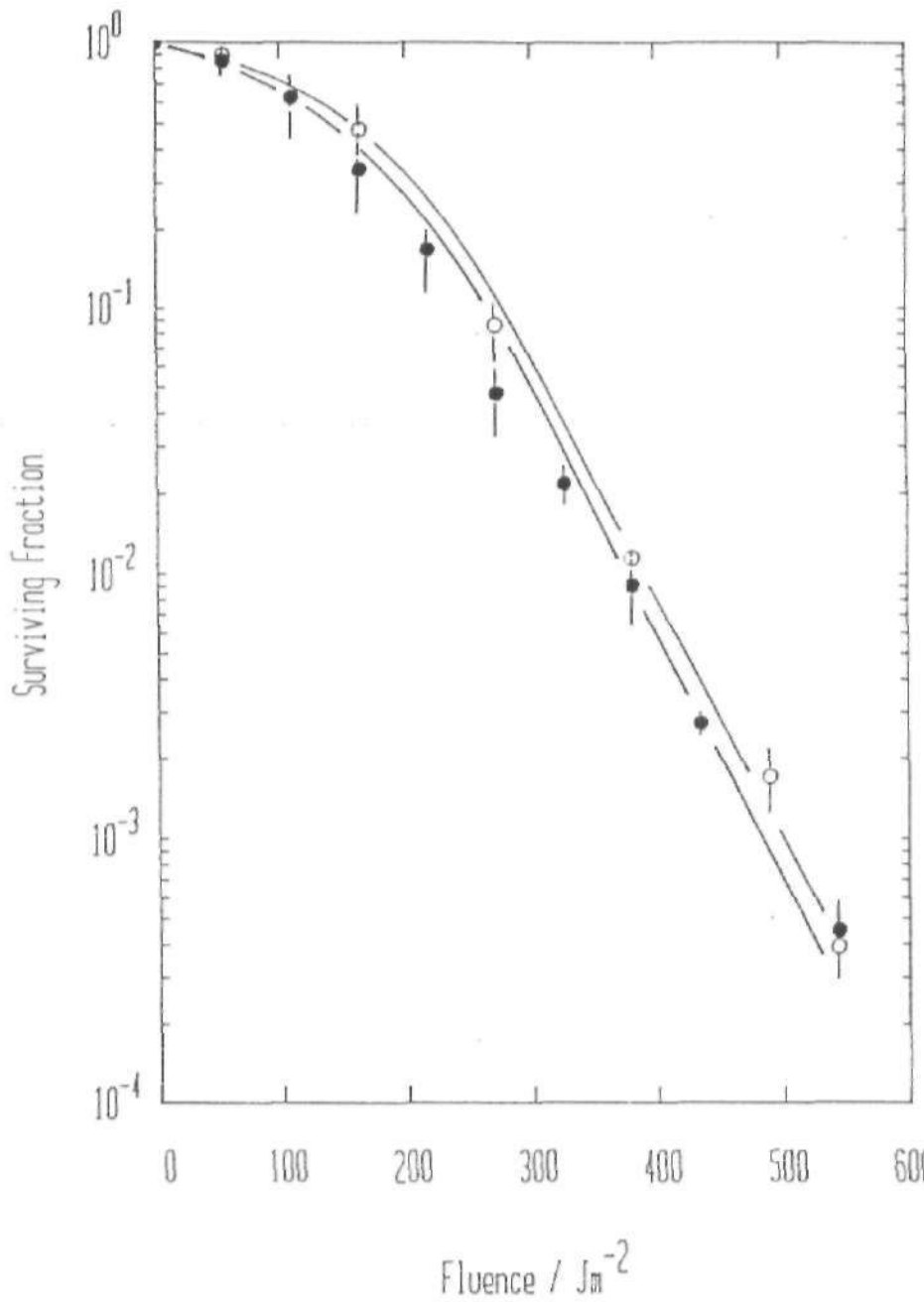


FIGURE 4.2: Inactivation of B. subtilis spores in suspension

- O Spore density: 1.2×10^6
- ● Spore density: 1.2×10^5

recovery is not 100% indicates that the bacterial cells adhere to polyethylene and/or that not all the cells in the initial population are viable.

4.2 Survival Curves of Spores in Suspension

To see the nature of the survival curve for suspension-irradiated spores so as to compare with irradiation in the dry state, the spores were irradiated while suspended in double distilled water. Different spore concentrations were used in order to observe the effect of spore concentration on inactivation.

Figure 4.2 shows the resulting survival curves and they are of the type described by the equation

$$f = n.\exp(-kF) \qquad (4.1)$$

for low survival levels (Alper, 1979)

where f is the surviving fraction

n is the extrapolation number

k is the inactivation constant

F is the mean lethal fluence

No. of irradiated cells (x 10^4)	Inactivation constant (k)	Extrapolation number (n)
1.2	0.012	9.0
12.0	0.012	10.0

Table 4.1: Inactivation parameters for suspension irradiation

As shown in the above table, the inactivation constant being a measure of sensitivity, remains the same for the different concentrations but the extrapolation numbers differ.

The difference in extrapolation number for the two samples may, in fact, be due to experimental error.

The extrapolation number represents the extent of the shoulder which in turn represents the ability of the cells to repair or accumulate damage (Alper, 1979). A comparison of the two curves in figure 4.2 shows a reduction of the shoulder for the smaller spore population.

4.3 Irradiation of spores on Polyethylene

The same concentration of spores was used in both cases so as to eliminate any shielding effect. Figure 4.3 shows the resulting curves whose parameters are given in Table 4.2

Moisture content of irradiated cells	Inactivation constant (k)	Extrapolation number (n)
Wet	0.0079	1.2
Dry	0.0081	2.0

Inactivation constant for the tailed part (same for the two curves) is 0.0033

Table 4.2 :Parameters for UV-survival of spore on Polyethylene

The results (figure 4.3) show tailed survival curves. The sensitivity of the spores appear to be enhanced by drying ,

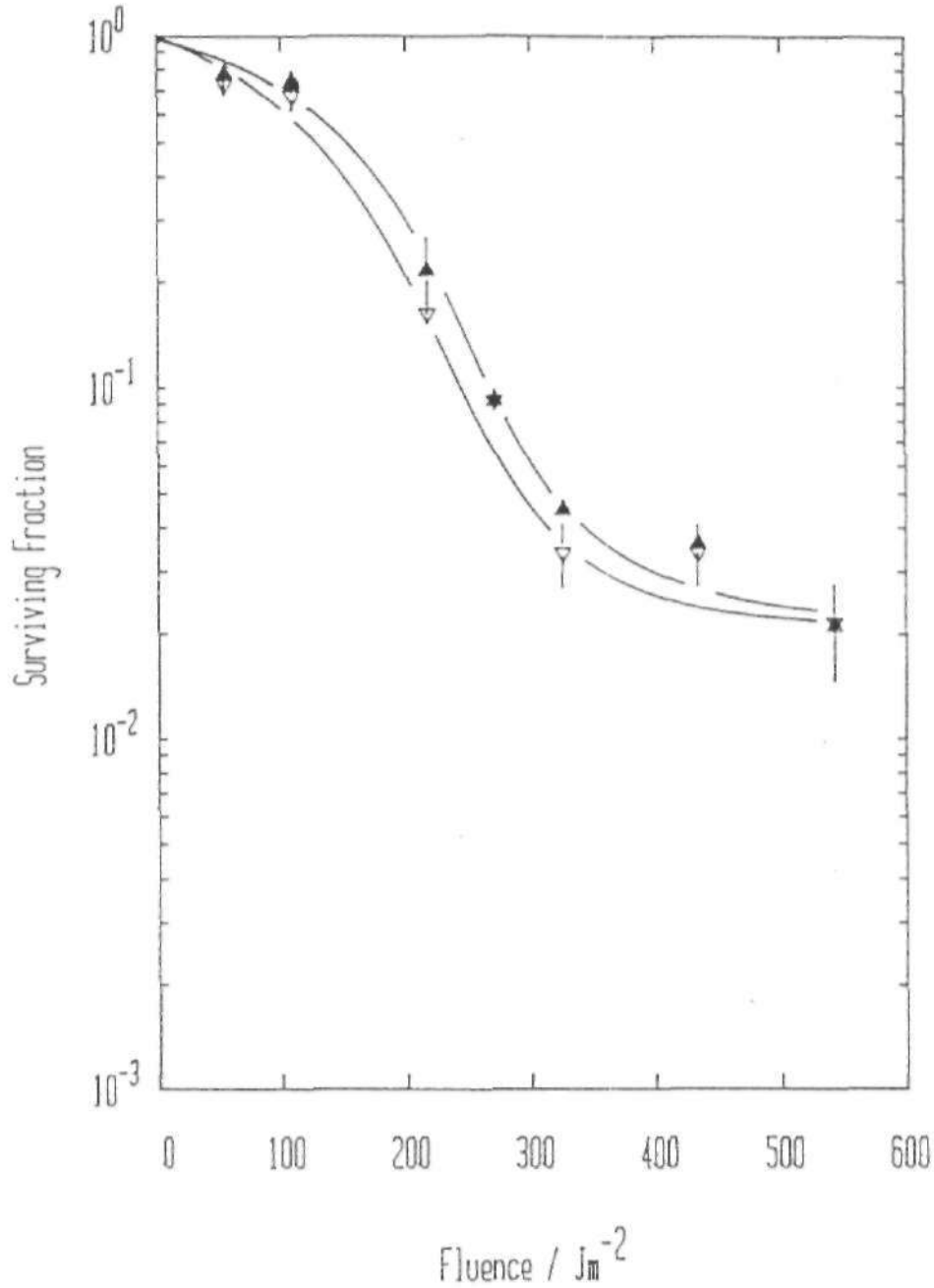


FIGURE 4.3: UV-Survival of B. subtilis spores on polyethylene

▲ Wet spores

▽ Dry spores

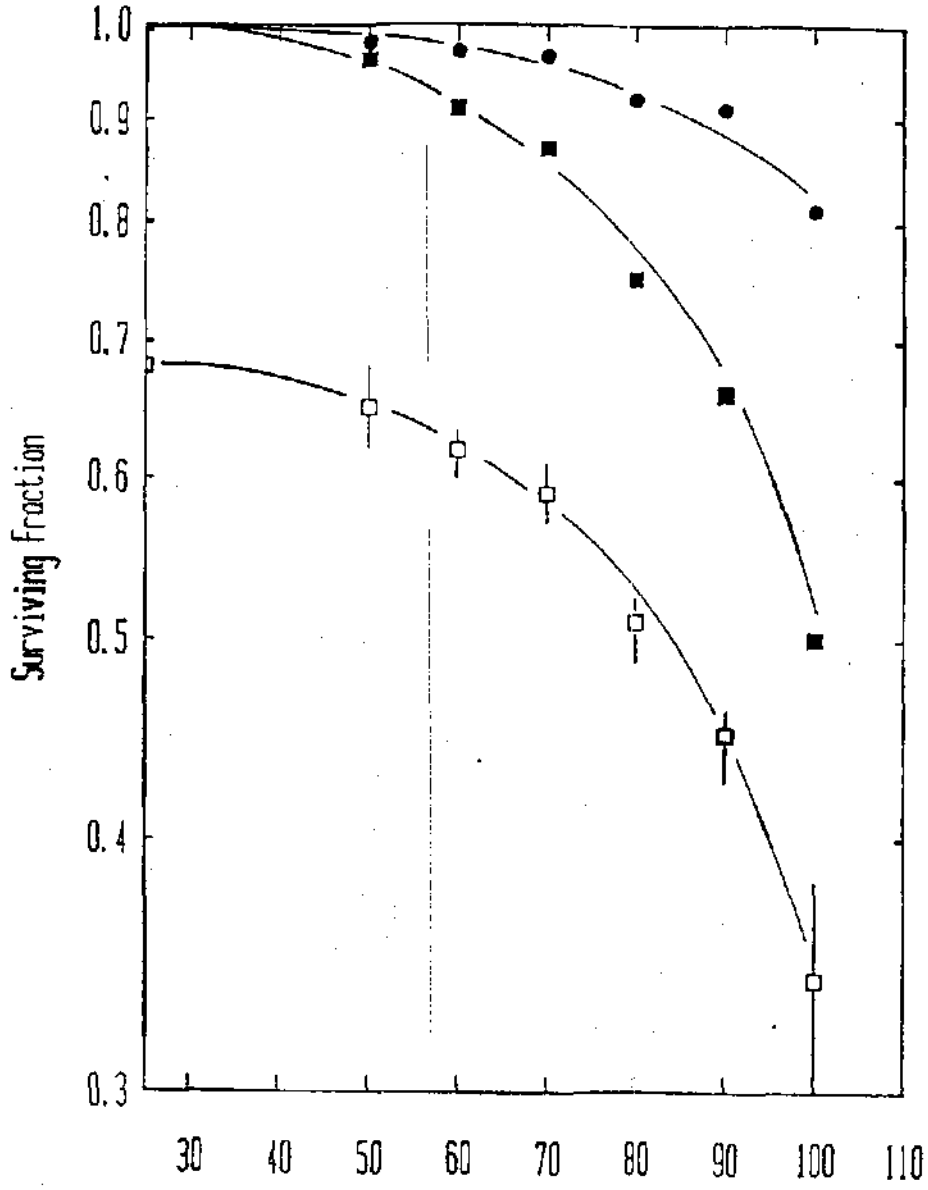


FIGURE 4.4: Action of UV and heat on B. subtilis spores

- ● Spores exposed to heat treatment
- □ Spores exposed to combined treatment and normalized with untreated control
- ■ Spores exposed to combined treatment and normalized with irradiated control

for low UV fluences. At the higher UV fluences, however, there appears to be little or no difference in sensitivity for the wet and dry spores. In this fluence region, the sensitivity is down by a factor of 2 compared with the low fluence region.

4.4 Effect of Heat on Spores

It was found that as far as inactivation is concerned, it made little difference whether the heat treatment was given before or after the UV irradiation. The resulting survival curves are shown in figure 4.4; the middle curve (plotted with dark squares) is obtained by normalising the combined treatment survival with the survival at 108.5 Jm^{-2} UV fluence.

The curves have practically the same general shape : a large shoulder up to 70°C-80 °C and a more pronounced inactivation effect above 80°C. This indicates that heating has little or no effect on the spores up to 70 °C-80 °C. The curve in the middle (normalised with the survival at 108.5Jm^{-2} UV) indicates the interaction between heat and UV-irradiation (the so-called synergistic effect) in spore inactivation. This shows that the degree of synergism is more pronounced in the 80°C-100°C region and also that even at 50 °C there is some amount of synergism.

4.5 Fluence-dependence of synergism

The UV fluence used was 217Jm^{-2} are shown in figure 4.5. Here, again, the curve in the middle (normalised with the survival at 217Jm^{-2} UV fluence) indicates the

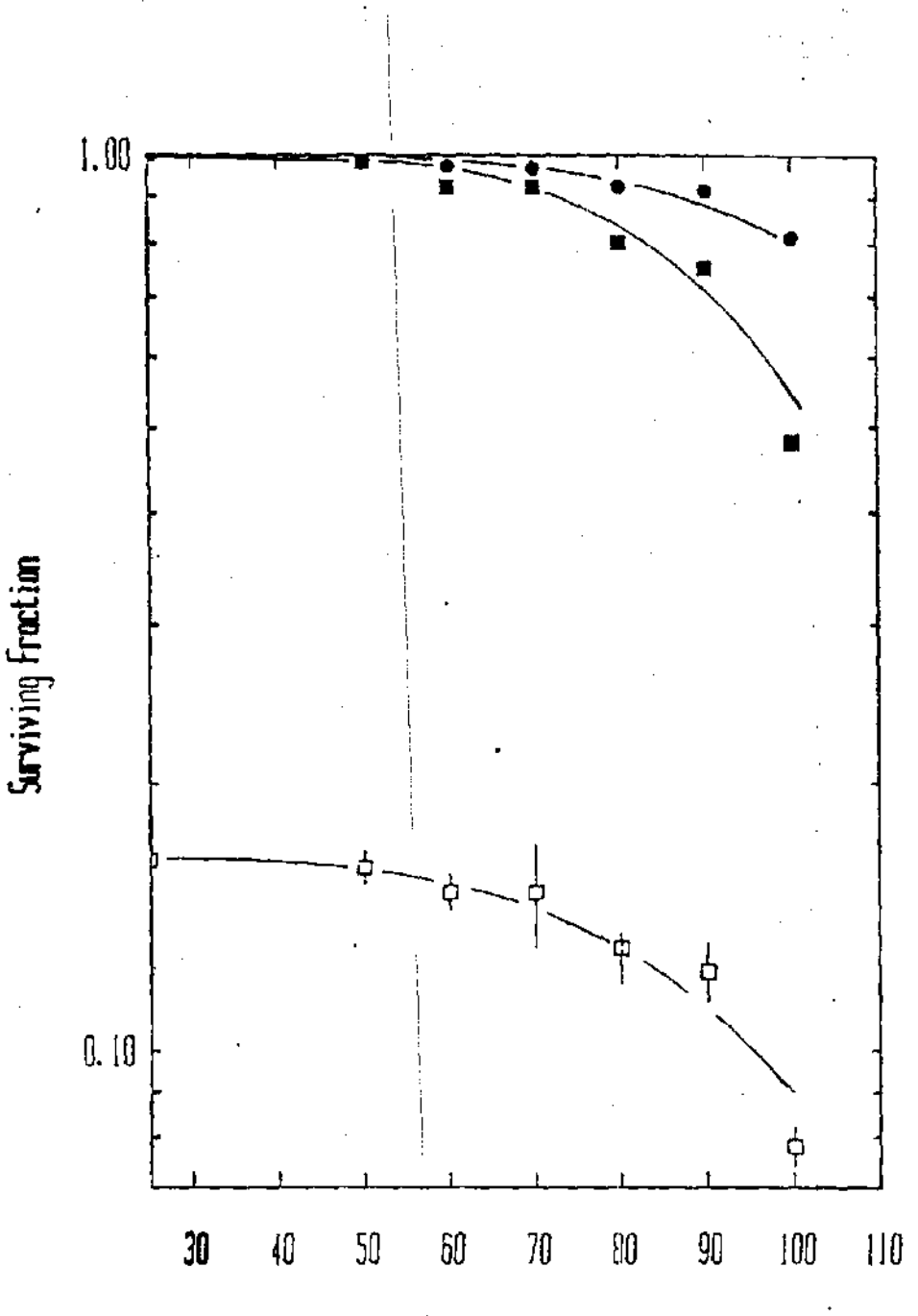


FIGURE 4.5: Combined action of UV and heat on dry spores

- ● Heated (but unirradiated) spores
- □ Spores exposed to combined treatment and normalized with untreated control
- ■ Spores exposed to combined treatment and normalized with irradiated control

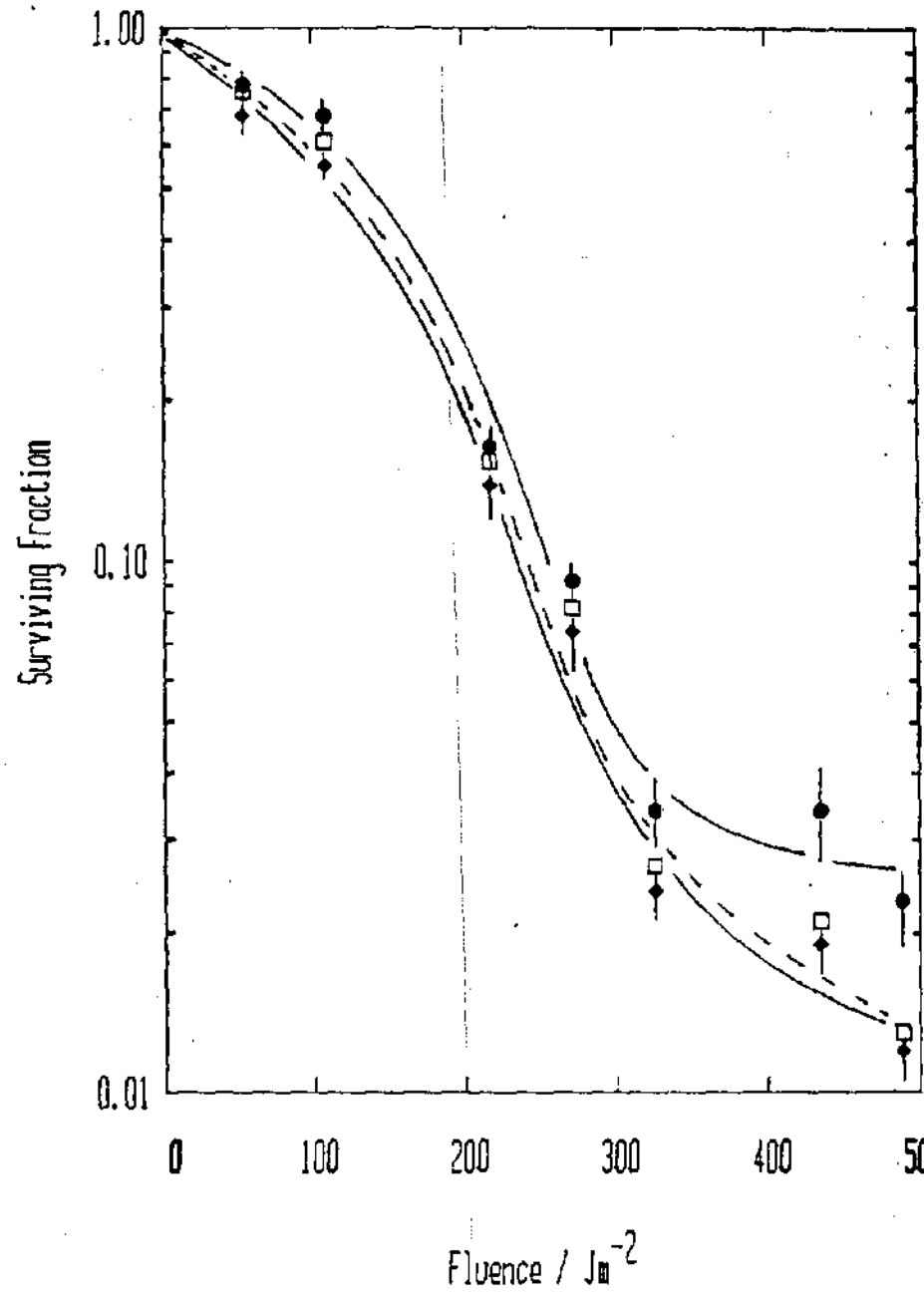


FIGURE 4.6: UV-Survival of mildly heated spores

- ● UV-irradiated spores
- ◆ Spores exposed to UV and heat (50°C)
- □ Spores exposed to combined treatment and normalized with heated control

synergistic effect at this fluence level. This shows that this effect is virtually the same as in the previous experiment. In the light of this result then, it can be stated that synergism is independent of UV fluence.

4.6 Synergism at 50°C

Dry spores on polyethylene were heated for 30 minutes at 50°C before being UV-irradiated and their survival was compared with that of spores exposed to UV irradiation alone. Here, again, it was found that it made practically no difference, on the inactivation effect, whether the heat was administered before or after irradiation. The results are shown in figure 4.6; the curve in the middle (with open squares) is obtained by normalising the combined treatment survival with the survival (of unirradiated spores) at 50°C.

Spore Treatment	Inactivation constant (k)	Extrapolation number (n)
Heat and irradiation	0.007	1.40
Irradiation alone	0.008	2.00

Table 4.3 : Inactivation parameters for survival of spores heated to 50°C.

The synergistic effect, the degree of which is indicated by the middle curve (with open squares) is more or less constant for the different fluence levels.

4.7 Effect of Prolonged Heating at 100°C

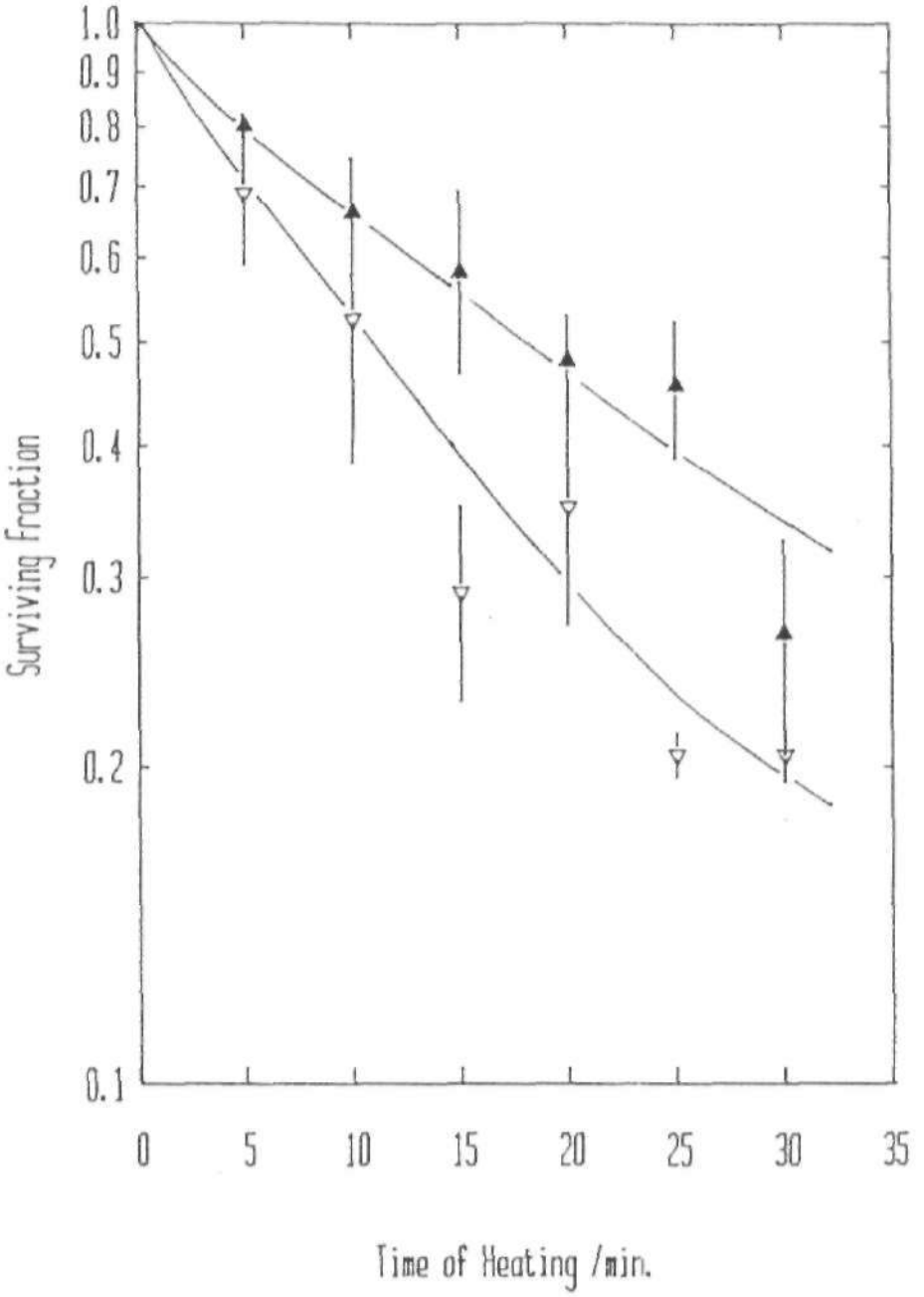


FIGURE 4.7: Spore inactivation at 100°C as a function of time

▲ Unirradiated spores

▽ Spores exposed to mild irradiation (54Jm^{-2})

The results of the preceding experiments, which indicate a more enhanced inactivation rate for spores heated to temperatures above 90°C provided the impetus for further examination of inactivation of spores subjected to prolonged heating at 100°C.

Here, two parallel inactivation experiments were carried out :the first with only heated spores and the second with spores subjected to both heating and mild UV irradiation ($54Jm^{-2}$). The results (see figure 4.7) show logarithmic inactivation with the UV-irradiated sample yielding a steeper curve - a more enhanced inactivation effect. A tendency towards tailing is also observed, especially for the UV-irradiated sample, after 25 minutes of heating.

4.8 Combined treatment (heat plus UV irradiation) at 85°C

In this experiment, spores (on polyethylene) were first heated for 5 minutes at 85°C before being irradiated with various UV fluences. This enabled the interaction between heat and UV radiation to be studied at this temperature which is about the point where the enzymes are inactivated. The survival was then compared with that of a sample which had not been heated.

The resulting curves (see figure 4.8) are exponentials but exhibiting a tail after the first decimal reduction. The UV-survival of the unheated sample shows a tail with more upward concavity than the heated sample.

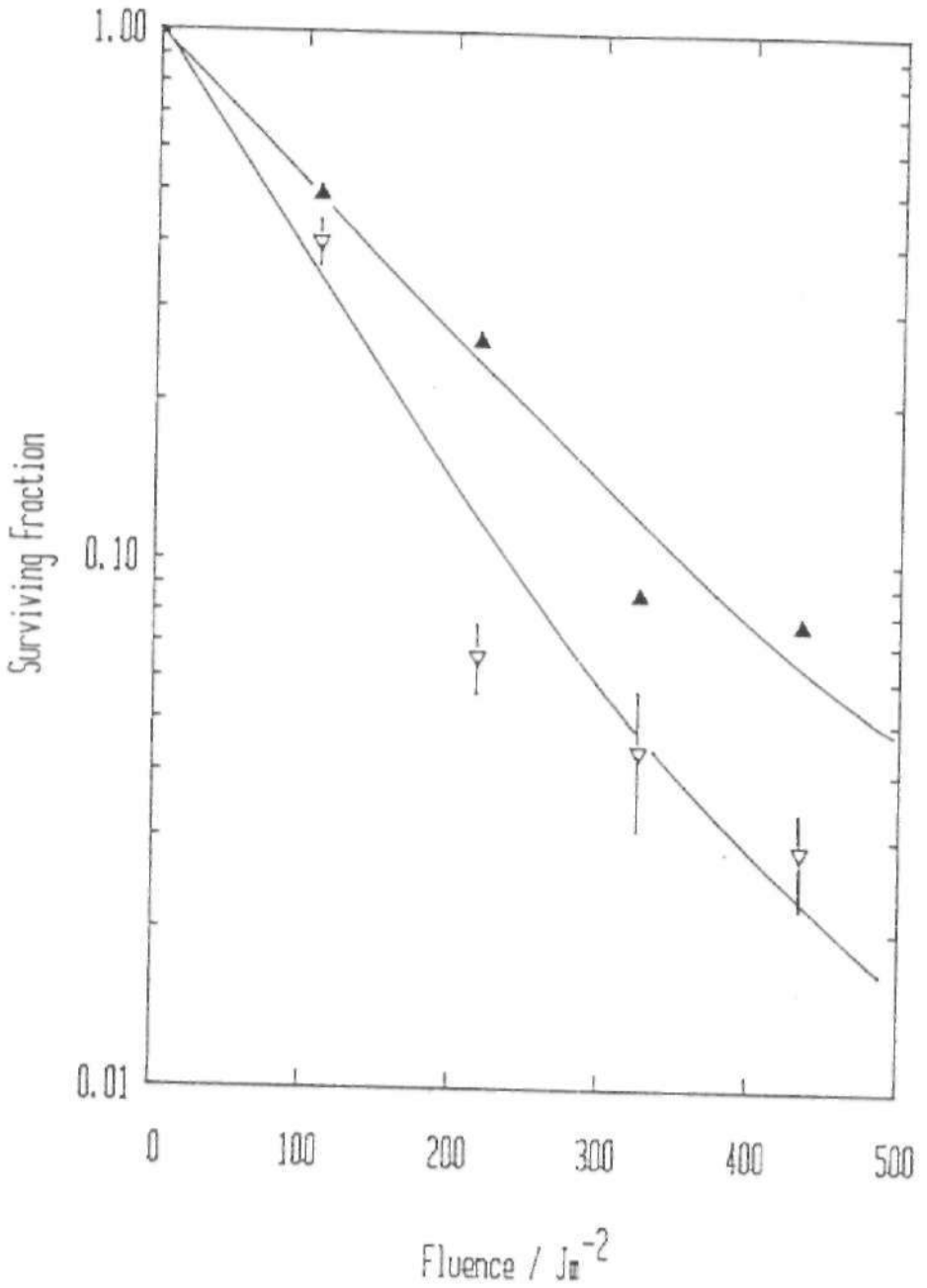


FIGURE 4.8: Combined action of UV and heat at 85°C

▲ Unheated spores

▽ Spores subjected to heating at 85°C

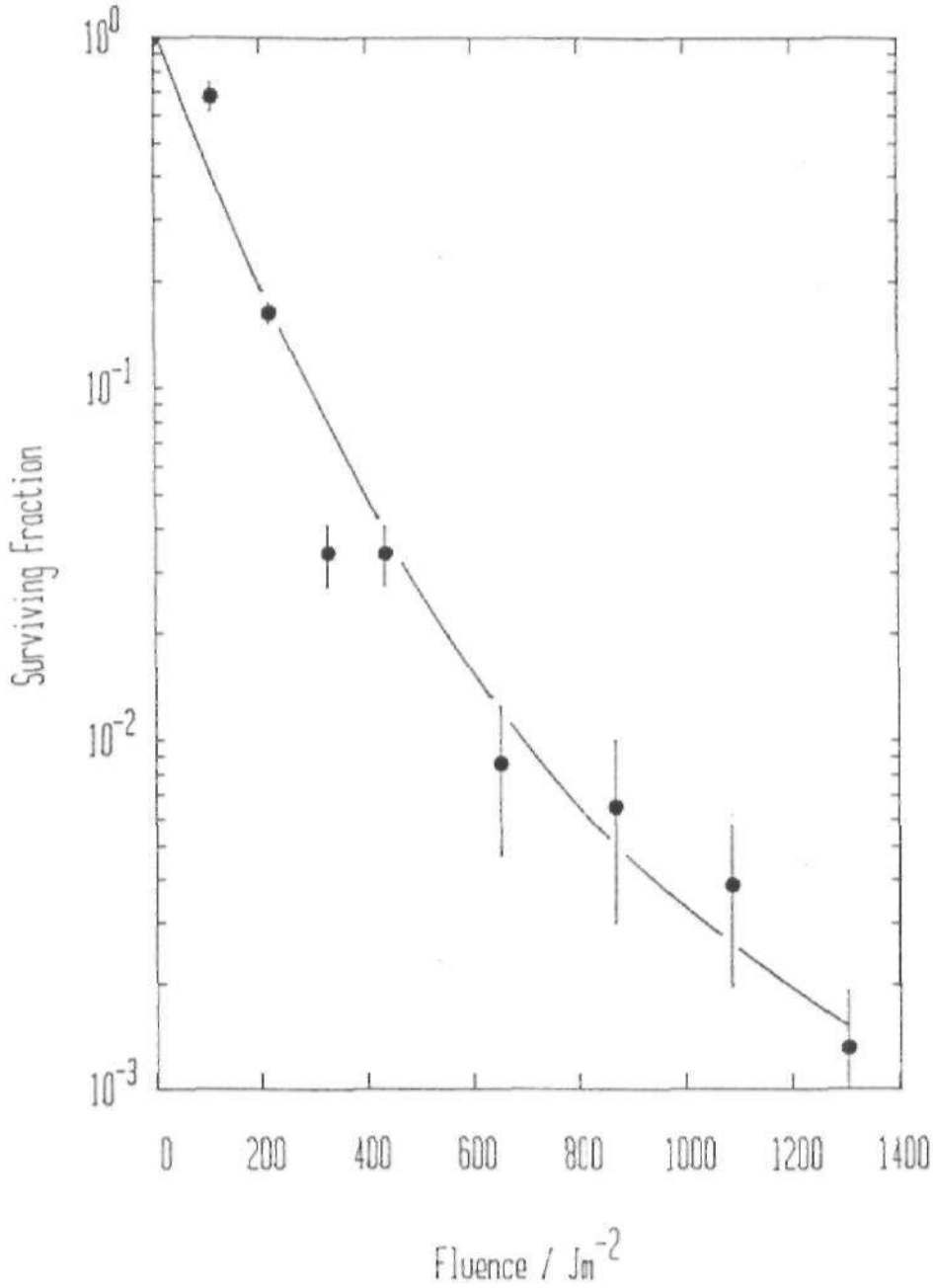


FIGURE 4.9: Inactivation of spores at elevated UV fluences

Treatment of spores	Inactivation constant (k)	Extrapolation number (n)
Heat plus UV	0.009	1.0
Only UV	0.007	1.0

Table 4.4 : Parameters for Spore survival at 85°C

4.9 Spore Survival at Elevated UV Fluences

The result shows a survival curve which is biphasic. The whole curve which has no shoulder and has an upward concavity, can be divided into two parts : one with an inactivation constant of 0.007 and the other with an inactivation constant of 0.004 (see figure 4.9).

4.10 Discussion

The important findings in this study may be summarised as follows :

(i) Electron microscopy indicates that Bacillus subtilis spores have some degree of adhesion to polyethylene surfaces leading to a plating efficiency of about 93%.

(ii) Inactivation of spore suspensions with 254nm UV yields shouldered survival curves which become linear at higher fluences with a mean lethal fluence of $83Jm^{-2}$.

(iii) Inactivation of wet and dry spores on polyethylene surfaces yields curves with the same general features : a reduced shoulder and higher mean lethal fluence (compared with irradiation of spore suspensions in glass Petri dishes) as well as a tailed part with an upward concavity.

(iv) Heating the spores before irradiating them produces the same result as irradiating them before subjecting them to heat treatment. When spores are subjected to a combined treatment of heating plus UV irradiation, their inactivation is more or less constant in the temperature range 50°C - 70°C but increases remarkably in the 80°C - 100°C range.

(v) There is evidence of some degree of interaction (synergism) between UV irradiation and heat, in the interaction process, and this appears more pronounced in the temperature range 80°C - 100°C. Further, this effect is found to be independent of fluence in the $108Jm^{-2}$ - $217Jm^{-2}$ fluence range.

(vi) Inactivation of dry spores of B. subtilis on polyethylene surfaces with elevated fluences results in a tailed survival curve with an upward concavity.

The phenomenon of adhesion of microorganisms to solid surfaces is well known. Zvyaginsev et al. (1971) reported high magnitude of adhesion of the bacterial genus Bacillus to several different surfaces. Using a specially developed method, they measured the adhesion forces of many microorganisms. It emerged that eventhough B. subtilis had the lowest number adhering to the given surface, after vigorous centrifugation, they nevertheless had one of the strongest adhesion forces measured. They also found that the cultivation time and age of the culture play some part in the ability of the microorganism to adhere to a given solid surface. Schenk and Kiefer (1987) reported some degree of yeast adhesion to glass surfaces which led to a

lowering of the plating efficiency. In this work, since only the 'live cell count' (leading to 100% plating efficiency) was used in all the experiments, the influence of the adhesion phenomenon on the results is circumvented.

The logarithmic inactivation of bacterial suspensions is more or less well established. Tyrell (1976) obtained a four decadic logarithmic inactivation for E. coli and Lyndberg et al. have published a shouldered survival curve which becomes linear at high fluences, for B. subtilis spores. The inactivation parameters they obtained compare favourably with values obtained in this work.

The effect of water on the sensitivity of cells exposed to radiation has been a matter for considerable debate. Bhattacharjee (1961) showed that vegetative cells of E. coli are less sensitive, by a factor of 3.4, to ionizing radiation after drying than are wet cells. Hutchinson et al. (1957) had obtained similar results with yeast cells. Dertinger and Jung (1969) explained these results as indicating an increase in the reaction of radicals formed from the water in biomolecules. On the other hand, Bücker and Horneck (1969) published a result quite contrary to that reported by the others.

However, in the present context, there can be no indirect effect of the type mentioned above, since far UV light does not form radicals from water. The slight difference in sensitivity observed may be due to some form of 'buffer' action by water against radiation in general, the exact mechanism of which is yet not very clear. It is

also possible that it may simply be a case of experimental error, since we are dealing with inert spores. In an attempt to explain away the "tailing" effect, Cerf and Metro (1977), claimed that the clumping of spores due to some property of the hairy coat is responsible. They claimed, further, that this artefact is sufficient to account for, not only the tailing phenomenon but also, the high variability of data obtained with the longest treatment times.

The "tailing" effect observed for spores inactivated on polyethylene, may, on the surface, be attributable to shielding. However, attempts to eliminate shielding by using lower spore concentrations failed to eliminate the tail. Also, UV inactivation experiments of dry B. subtilis on glass surfaces, reported by Lindberg (1987), using identical methods as was used in this work showed no such tails. It may be well to mention here that tailed survival curves with an upward concavity was reported by Ito et al. for Clostridium spores; a number of other workers in the field have also reported similar results (see section 2.10)

In the present work, since the tailing effect is common to all the curves obtained, it may be worthwhile to study further the possible causes. It is possible that the less sensitive fraction represents mutations of the original spore population. It is, also, not beyond the realms of probability that there may be some interaction, chemical or otherwise, between the spores and polyethylene material which confers some degree of protection to a fraction of the spores.

Finally, on the combination (UV-irradiation plus heat) treatment, it must be said that more detailed work has to be done before any conclusive statements can be made. Many workers in the field have explored various other combinations in a bid to optimize the inactivation effect. Waddle and Renninger (1975), Toledo et al. (1973), Bayliss and Waites (1979), Cerf and Metro (1977) all reported enhanced killing of B. subtilis spores when UV irradiation is combined with hydrogen peroxide. Swartling and Lindgren (1968) combined hydrogen peroxide with heat and reported some synergistic enhancement of the killing effect on B. subtilis. Tyrrell (1976), examined the synergism between different wavelengths of UV radiation (254, 334 and 365nm) as well UV and heat in the inactivation of the bacteria E. coli. In the present work, the most that can be said is that there is evidence of some degree of synergistic enhancement by combining UV and heat.

4.10 Conclusion

As it is indicated above, some work still needs to be done to determine the correct and "unequivocal" nature of the survival curve of UV-irradiated B. subtilis on polyethylene. If, indeed, the observed tail represents a more resistant fraction of the spores, it becomes important for sterilization puposes; the possible cause of the tail has to be identified with a view to its eradication. However, it emerged (see section 4.2) that decreasing the spore concentration increased the effectiveness of UV and heat so that packaging, which usually has a low spore count

of about 100 - 300m (Swartling and Lindgren, 1962), might be sterilized with a less severe treatment. That is to say that it should be easier to produce the 99.99% kill needed for sterilisation with mild combination of UV and heat. The fact remains, however, that for any given packing material, the exact nature of survival curves of B. subtilis on it must be known in order to be able to predict the severity of the treatment needed for sterilisation.

The foregoing notwithstanding, the experiments reported in this study, have shown that a combination of UV and heat is more effective in the inactivation of B. subtilis than a simple addition of the two processes. On the whole, depending on the nature of the food product whose packaging is to be sterilized, a combination treatment of UV and heat may also turn out to be less "messy" and more economical than that of hydrogen peroxide and heat or hydrogen peroxide and UV.

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Fluence (Jm^{-2})	Surviving Fraction ($x10^{-2}$)	Fluence (Jm^{-2})	Surviving Fraction ($x10^{-2}$)
0.0	100	0.0	100
54.25	85.2 ± 3.0	54.25	89.7 ± 1.5
108.50	62.5 ± 1.7	162.75	47.4 ± 1.8
162.75	33.6 ± 1.2	271.25	8.6 ± 0.6
217.00	16.7 ± 0.61	379.75	1.14 ± 0.02
271.25	4.72 ± 0.41	488.25	0.17 ± 0.046
325.50	2.16 ± 0.36	542.50	0.039 ± 0.009
379.75	0.90 ± 0.11		
434.00	0.27 ± 0.028		
542.50	0.045 ± 0.0037		

DATA FOR FIGURE 4.2

Fluence (Jm^{-2})	Surviving Fraction ($x10^{-2}$)	Fluence (Jn^{-2})	Surviving Fraction ($x10^{-2}$)
0.0	100	0.0	100
54.25	85.0 ± 4.6	54.25	73 ± 0.7
108.50	35.0 ± 2.0	108.50	68 ± 6.4
217.00	10.0 ± 0.2	217.00	16.3 ± 1.1
271.25	7.4 ± 0.1	271.25	9.2 ± 0.0
325.50	2.4 ± 0.5	325.50	3.40 ± 0.7
434.00	3.0 ± 0.06	434.00	3.39 ± 0.67
488.25	2.3 + 0.02	542.5	2.15 + 0.32

DATA FOR FIGURE 4.3

Fluence (Jm^{-2})	Surviving Fraction ($\times10^{-2}$)	Fluence (Jm^{-1})	Surviving Fraction ($\times10^{-1}$)
0.0	100	0.00	100
54.25	85.2 ± 3.0000	54.25	89.7 ± 1.500
108.50	62.5 ± 1.7000	162.75	47.4 ± 1.800
162.75	33.6 ± 1.2000	271.25	8.6 ± 0.600
217.00	16.7 ± 0.6100	379.75	1.14 ± 0.020
271.25	4.72 ± 0.4100	488.25	0.17 ± 0.046
325.50	2.16 ± 0.3600	542.50	0.039 ± 0.009
379.75	0.90 ± 0.1100		
434.00	0.27 ± 0.0280		
542.50	0.045 ± 0.0037		

DATA FOR FIGURE 4.2

Fluence (Jm^{-1})	Surviving Fraction ($\times10^{-2}$)	Fluence (Jm^{-2})	Surviving Fraction ($\times10^{-2}$)
0.0	100	0.0	100
54.25	85.0 ± 4.60	54.25	73 ± 0.70
108.50	35.0 ± 2.00	108.50	68 ± 6.40
217.00	10.0 ± 0.20	217.00	16.3 ± 1.10
271.25	7.4 ± 0.10	271.25	9.2 ± 0.00
325.50	2.4 ± 0.50	325.50	3.40 ± 0.70
434.00	3.0 ± 0.06	434.00	3.39 ± 0.67
488.25	2.3 ± 0.02	542.5	2.15 ± 0.32

DATA FOR FIGURE 4.3

DATA FOR FIGURE 4.4

Temp. (°C)	Surviving Fraction (x10-²)	Temp. (°C)	Surviving Fraction (x10-²)
25	100 ± 0.00	25	100 ± 0.1
50	64.0 ± 1.7	50	98 ± 0.1
60	62.0 ± 1.0	60	96 ± 0.30
70	47.0 ± 2.0	70	95 ± 1.0
80	38.0 ± 1.3	80	93 ± 2.0
90	29.0 ± 0.7	90	91 ± 1.0
100	34 ± 1.0	100	87 ± 4.0
Heat + UV irradiation (normalized with irradiated control)		Heat treatment only	