IMMUNOLOGICAL STUDIES OF THYMINE DIMER QUANTITATION

by

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PREFACE

This thesis presents work carried out by the author and has not been submitted in part, or in whole, to any other university. Where use has been made of the work of others, it has been duly acknowledged in the text.

The work described in this thesis was carried out in the Department of Chemistry and Applied Chemistry, University of Natal, King George V Avenue, Durban, 4001, from January 1991 to August 1992 under the supervision of Prof. L.F. Salter.

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ABSTRACT

Ultraviolet irradiation of DNA induces the formation of a number of mutagenic lesions. The most prolific of these is the cis-syn thymine dimer (formed maximally at 260 nm) and this has been implicated in the reaction pathways that lead to ultraviolet-induced carcinogenesis.

In order that the molecular events underlying these neoplastic events be understood, it is imperative that the thymine dimers formed in ultraviolet-irradiated thymine containing systems be quantitated. In this laboratory, dimer quantitation is performed using reverse phase high performance liquid chromatography (HPLC) with ultraviolet (UV) detection and the data obtained has allowed a kinetic mechanism for lesion formation to be proposed.

Such studies have used *in vitro* thymine containing substrates (aqueous thymine, thymidine, thymidylyl-3',5'-thymidine, calf thymus DNA and pUC19 plasmid DNA) to generate the thymine dimer using UV irradiation. With the planned extension of this research to *in vivo* cellular systems (where DNA and hence thymine concentrations are intrinsically less than those of *in vitro* systems), a more sensitive technique for thymine dimer quantitation is required.

An immunological approach to providing this technique was chosen. Here, UV-irradiated DNA was injected into rabbits whose immune system mounted a response (i.e. antibody production) to the UV-DNA antigen. Blood was drawn from the rabbits at regular intervals to obtain the antibodies. The technique of immunoblotting was chosen and developed to allow detection of the thymine dimer antigen. This involved

the reaction between the UV-DNA antigen, the primary antibody (generated by the rabbit) and a secondary antibody conjugated to an enzyme, all of which were immobilized on a commercially available membrane system.

Detection and quantitation of the immune complex immobilized on the membrane was performed using the technique of enhanced chemiluminescence. Upon addition of a chemiluminescent substrate (luminol) to the immune complex, the horseradish peroxidase enzyme catalysed the reaction of luminol, with one of the products being light of 425 nm to 430 nm. This light impinged on a luminescence film which was developed and printed using standard photographic techniques. The use of dilutions of the primary antibody in the immunoblotting protocol with enhanced chemiluminescent detection, allowed correlations of antibody dilutions with UV-DNA antigen to be made.

This immunoblotting technique with enhanced chemiluminescent detection has been used successfully in detecting thymine dimer lesion formation at levels currently above the detection limit of the HPLC. It has also been used successfully in detecting and quantitating thymine dimers at levels undetectable by the HPLC. To this end it has proved to be 4000 to 8000 times more sensitive than the chromatographic technique.

Any immunological technique requires that the antibody of interest be purified and characterized. Here, purification of the crude serum was performed using the classical technique of ammonium sulphate precipitation of proteins. As an alternative technique, affinity chromatography was performed on the crude serum using a Memsep 1000 affinity chromatography cartridge attached to a preparative HPLC system. Chromatographic data illustrating this purification are given. Characterization of the UV-DNA antigen was performed by considering the specificity of the antibody

response in the laboratory animal.

Support for the kinetic mechanisms previously proposed for pyrimidine dimer formation in DNA is also given in this work. Calf thymus DNA was irradiated and dimer yields obtained by immunoblotting. These were used in the computer programme CAKE together with the previously determined rate constants to determine simulated dimer yields. A good agreement between experimental and simulated data indicated the validity of the mechanism at a DNA concentration of 0.025 mg/ml.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
c-s	cis-syn
(6-4)	pyrimidine-(6-4)-pyrimidone
UV	ultraviolet
HPLC	high performance liquid chromatography
acp	acetophenone
E. coli	*Escherichia coli*
UV-DNA antigen	ultraviolet-irradiated DNA antigen
ab	antibody
s/c	subcutaneous
i/m	intramuscular
i/v	intravenous
i/v T	intravenous terminal
PBS	phosphate buffered saline
PBS-T	Tween-20 dissolved in phosphate buffered saline
PEG	polyethylene glycol
PVDF	polyvinylidene difluoride
ELISA	enzyme linked immunosorbent assay
MBSA	methylated bovine serum albumin
HRP	horseradish peroxidase

ECL	enhanced chemiluminescence
CAKE	computer analysis of kinetic equations

CONVENTIONS FOLLOWED

Where units and symbols used in this thesis are not IUPAC or SI approved, they are those most commonly used in the field. This comment is most relevant in those sections pertaining to DNA sample preparation where mg/ml or μg/ml concentration units are used. Antibody dilutions (e.g. a one in one thousand dilution) are expressed as 1:1000 where extensive use has been made of μl volume units during the explanation of the antibody dilutions and elsewhere in the immunoblotting protocol.

Lines drawn through points on a graph are best fit lines, except in Chapter 6 where simulated data is superimposed on experimental data points.

CHAPTER 1 INTRODUCTION

This thesis describes an immunological technique for the detection and quantitation of cyclobutane thymine dimers in ultraviolet irradiated deoxyribonucleic acid (DNA). Absorption of photons of ultraviolet (UV) irradiation by cellular DNA leads to thymine dimer formation and this event is implicated in the reaction pathways that effect UV-induced carcinogenesis. In these laboratories thymine dimer quantitation is performed using reverse phase high performance liquid chromatography with UV detection. However, this chromatographic technique cannot provide the sensitivity required for the quantitation of thymine dimer at cellular levels where dimer concentrations are inherently less than those of *in vitro* thymine containing systems. An immunological technique for thymine dimer quantitation was thus developed and is presented in this thesis.

This chapter briefly reviews features of DNA photochemistry, considering primarily cyclobutane thymine dimer formation (Section 1.1), and then considers immunological aspects of thymine dimer photochemistry (Section 1.2).

1.1 THE EFFECTS OF ULTRAVIOLET IRRADIATION ON DNA

Deoxyribonucleic acid is the store of genetic information for all living cells. The DNA macromolecule is thus responsible for the transmission and expression of genetic information and thereby controls cellular functioning. Its structure was established by Watson and Crick[1] in 1953 as being two polynucleotide chains plectonemically twisted about the same central axis to form a double helix[2] (see Figure 1.1.[3]). Each strand of the helix is constructed from the four nucleic acid bases (adenine, thymine, guanine and cytosine) bound to a sugar-phosphate backbone by

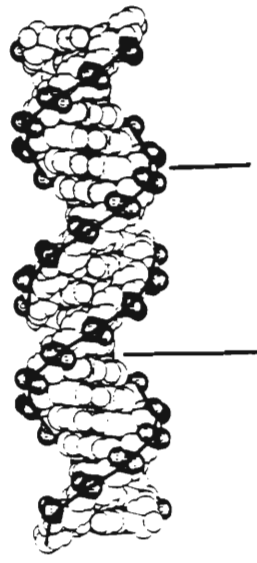


Figure 1.1. Space filling model of a portion of the double helical DNA molecule[3].

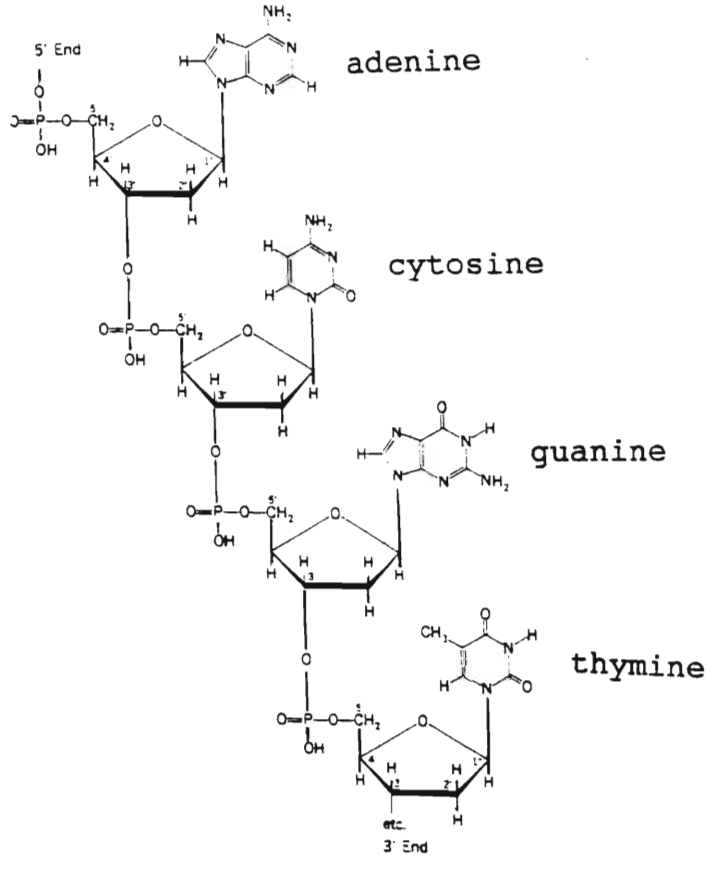


Figure 1.2. A segment of a single polydeoxyribonucleotide strand illustrating the phosphate-deoxyribose backbone with attached nitrogenous bases[4].

phosphodiester linkages in 3'-5' or 5'-3' configurations (see Figure 1.2.[4]). The nucleic acid bases are located towards the centre of the double helix and are arranged in such a fashion that a pyrimidine (thymine or cytosine) moeity of one strand pairs with a purine (adenine or guanine) base on the other strand. The helix is stabilized by interstrand hydrogen bonding between the nucleic acid bases (see Figure 1.3.[2]) and intrastrand stacking forces between adjacent nucleotides. X-ray studies on DNA fibres of synthetic polymers and natural sequences suggest that the DNA molecule can adopt one of several conformations (A, B or C) depending on the nature of its environment. Conformation B is that found most commonly in aqueous solutions of low ionic strength (i.e. in living systems).

In vivo DNA exists as free molecules within the cytoplasm of prokaryotes or as protein associated molecules within the nucleus of eukaryotes. It is the sequence of nucleic acid bases within the nuclear material which encodes the genetic information to be transferred sequentially from the DNA to form proteins. This flow of information involves transcription of a single DNA strand to form RNA, and translation of the RNA to synthesize amino acid chains which adopt conformational structures as proteins. Aberrations of the encoded sequence of nucleic acid bases leads to the eventual synthesis of proteins and enzymes incapable of functioning within the cellular environment. At the molecular level, such alterations of the genetic code may be manifest as an inability by enzymes to control DNA replication, RNA synthesis or protein synthesis, and this may lead to tumour development.

Alterations in base sequence may be brought about by a variety of chemical, viral or physical sources. Chemical change is occasioned by electrophilic reagents which covalently interact with DNA components[5] and form DNA adducts which can interfere with the flow of genetic

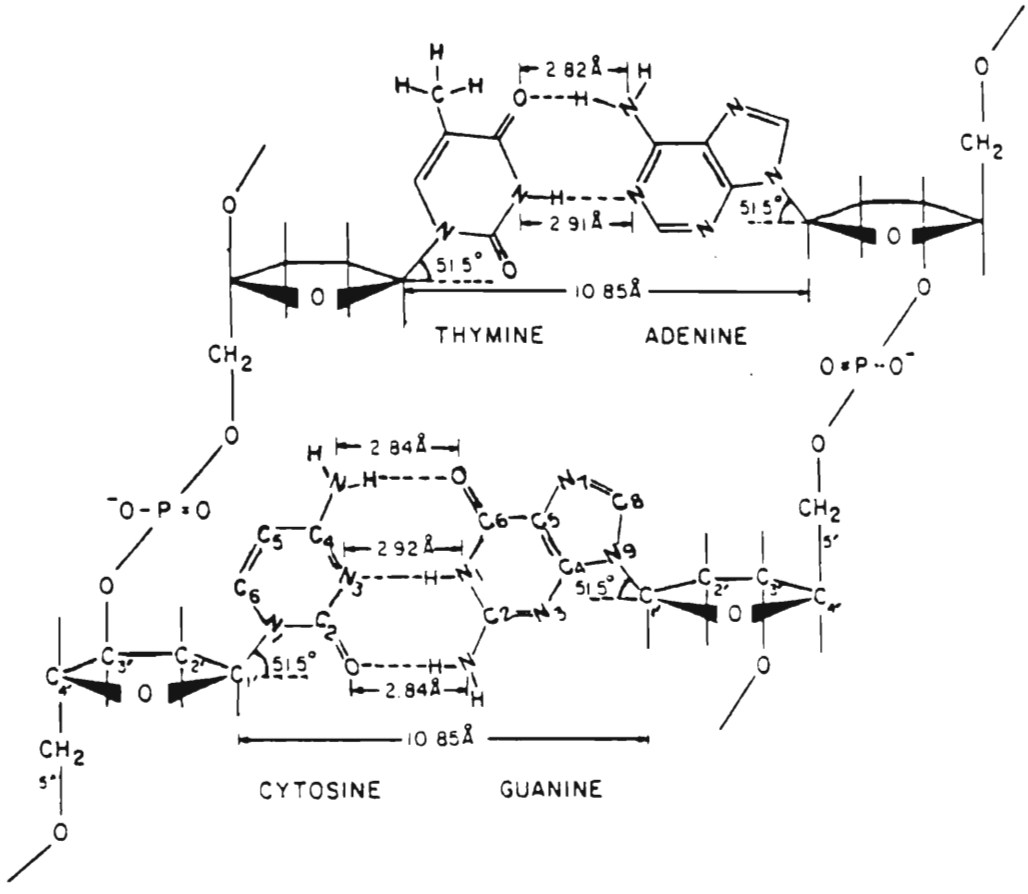


Figure 1.3. Molecular detail of a portion of a double helical DNA strand, illustrating specifically the interstrand hydrogen bonding[2].

information. Viral damage is caused by the injection of viral DNA into the host cell where its incorporation into DNA also interferes with the fidelity of transcription and translation. Physical damage to DNA may result from exposure to mechanical forces (e.g. shearing) or radiation. Radiation damage is associated with exposure to any part of the electromagnetic spectrum, from gamma to radiowaves[6], with each type of radiation band inducing different types of damage to the DNA macromolecule. It is this last mentioned damage mechanism (radiation damage) which is of concern here, in particular DNA damage formed by interaction with ultraviolet (UV) radiation.

Exposure of the skin to UV irradiation (100 to 400 nm) induces both physiological and pathological events. Several lines of evidence implicate DNA as the target for most of the biological effects of UV, such as mutation, lethality and transformation. The action spectrum for cell killing, mutation and transformation of golden hamster embryo fibroblasts[7] and human cells[8] are similar to the DNA absorption spectrum as well as the action spectrum for cyclobutane pyrimidine dimer induction (see later). Further evidence in support of this link between UV radiation and DNA comes from studies of individuals with the genetic disorder xeroderma pigmentosum. These individuals are extremely sensitive to sunlight and frequently develop skin cancer as a result of UV exposure. Their somatic cells are hypersensitive to killing by UV radiation[9] and this extreme sensitivity has been attributed to a defect in the repair of UV-induced pyrimidine dimers. These studies suggest DNA to be a target in UV-carcinogenesis, with the pyrimidine dimer being responsible for at least some of the abovementioned biological effects.

A number of studies [10-12] have investigated the link between pyrimidine dimer formation and skin cancer and have indicated that there is at least a causal relationship

between induction of pyrimidine dimers and UV-carcinogenesis. Further work has also implicated other non-dimer photoproducts in UV-induced lethality and mutagenesis. The results obtained are primarily dependant on the UV wavelengths used for analysis purposes. Each band (A, B or C) of the UV spectrum exhibits different biological effects. Ultraviolet A (UVA, 320 nm to 400 nm) and ultraviolet B (UVB) from 280 nm to 320 nm are biologically relevant because they penetrate the stratospheric ozone layer. A filtering effect due to this protective ozone layer prevents much of the UV wavelengths of less than 290 nm from reaching the earth's surface.

For experimental reasons (large quantum yields at UVC wavelengths gave large product yields), the earliest work on the effects of UV radiation on DNA concentrated on the UVC range where the formation of pyrimidine adducts and cyclobutane dimers could be directly implicated in the reaction pathways that lead to carcinogenesis. As more powerful UV sources and better analytical techniques were developed, these investigations were extended to UVA and UVB regions. In the UVC range, primarily at 260 nm, the cyclobutane pyrimidine dimer (shown in Figure 1.4.[13]) was found to be induced at significant levels, with the pyrimidine (6-4) pyrimidone photoproduct being detected at lower concentrations. Induction of cyclobutane pyrimidine dimers by 365 nm light on extracted purified DNA is poor in contrast with the high yield of dimers generated in intact *E. coli* bacteria. This suggests that an endogenous sensitizer (see later) may mediate pyrimidine dimer production at wavelengths greater than 320 nm[14]. In the UVA range above 365 nm, the most prevalent lesions are single stranded breaks and DNA-to-protein cross-links[15], which may interfere with the fidelity of the genetic code. UV light can be lethal to cells and was shown to be cytotoxic in human cells, possibly at wavelengths as long as 405 nm[16].

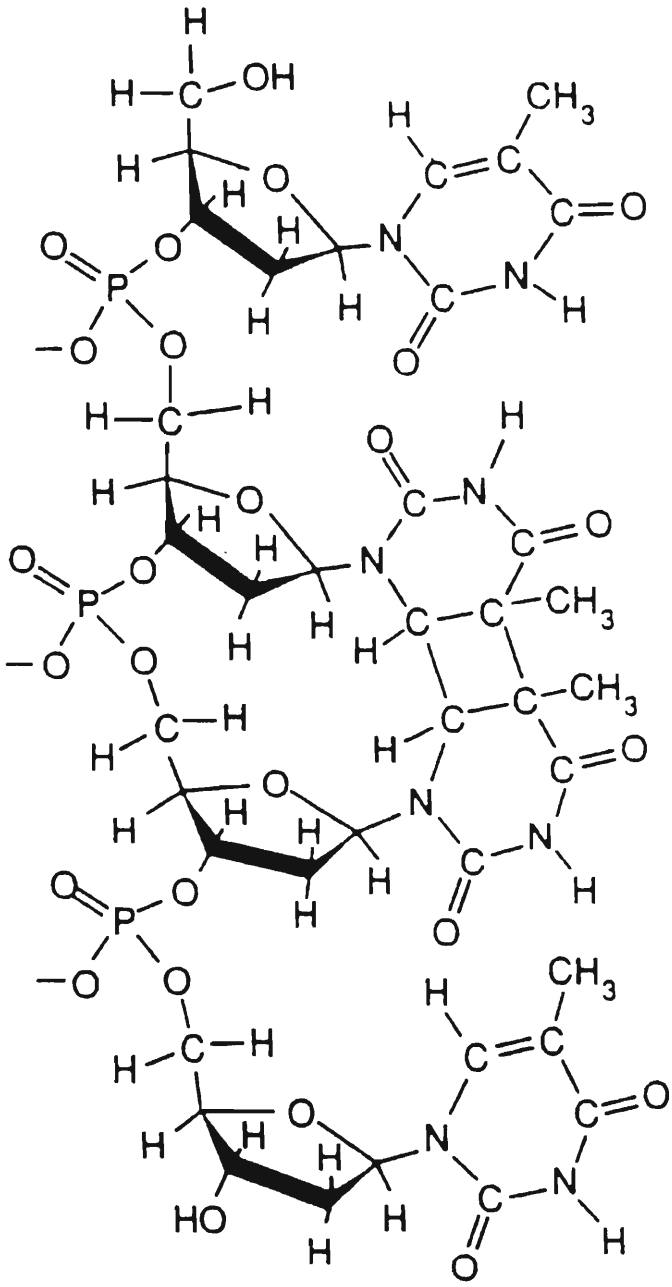


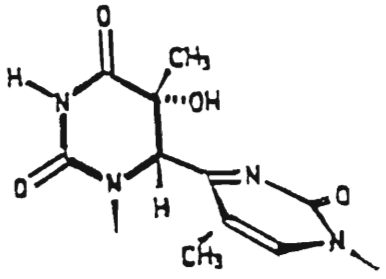
Figure 1.4. A segment of a single DNA strand illustrating the thymine dimer[13].

As mentioned above, damaging radiation in the UVB and UVC regions is absorbed by stratospheric ozone but epidemiological data suggests that radiation can penetrate the stratospheric ozone layer and impinge on the epidermal layers of the skin to cause the induction of skin cancerous tumours[17]. Hence UVB radiation has been implicated in the aetiology of squamous and basal cell carcinoma (associated with long term sun exposure) and malignant melanoma, the most feared form of skin cancer which has been linked to blistering sunburn in early life[18]. Although UVA irradiation is one thousand fold less effective than UVB in producing skin redness (erythema) upon irradiation, its predominance in the solar energy reaching the earth permits UVA to play an important role in contributing to harmful effects of UV radiation. Indeed its significance is now thought to be much greater than previously suspected. UVA is implicated in dermal tissue alterations[19] and tumours[20], and has been reported to produce chemical phototoxic and photoallergic reactions[21] and to be involved in immune supression due to both inhibition of Langerhans cells and induction of supressor T-cells. (These immunological perspectives will be discussed fully in Section 1.2.)

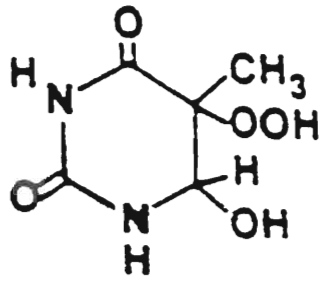
As mentioned earlier, cyclobutane pyrimidine dimers and the pyrimidine (6-4) pyrimidone photoadduct are implicated as the most significant lesions formed in UV-irradiated DNA and a wealth of literature exists to support this. The initial isolation and identification of the cis-syn cyclobutane dimer from irradiated frozen aqueous solutions of thymine in 1960 by Beukers and Berends[22] was followed by confirmation of its presence in UV-irradiated DNA by Weinblum[23] and Wacker et al.[24], whilst the 6-4'-[pyrimidin-2-'one] thymine photoproduct was first described by Wang and colleagues[25] in the late 1960's after isolation from UV-irradiated and acid hydrolysed DNA. Early studies also identified the cis-syn cyclobutane cytosine

dimer and the mixed cis-syn thymine-cytosine dimer in UV-irradiated DNA molecules. The cis-syn isomer is favoured because it involves a relatively unhindered coupling of adjacent thymine or cytosine residues along the DNA strand. (The trans-syn thymine dimer is formed in denatured DNA where strand separation permits greater flexibility of adjacent nucleotides.) The 5-thyminyl-5,6-dihydrothymine (formed at low relative humidities) and pyrimidine trimers were also identified in UV-irradiated DNA, and thymine hydroperoxides and thymine glycols were isolated from irradiated aqueous thymine reactions. Despite the identification of these photoproducts from irradiated systems, only the cis-syn pyrimidine dimers and the 6-4 photoproducts are generated in sufficiently high concentrations to make them amenable to correlations with biological damage. Figure 1.5. illustrates the structures of some of the abovementioned photoproducts.

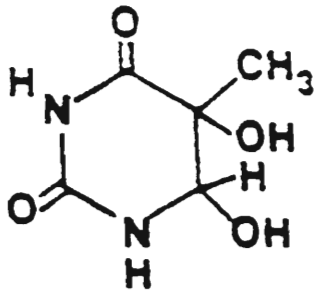
It was primarily because the cyclobutane pyrimidine dimer was stable and was produced in large amounts that experimental investigations concerning its correlation with UV photodamage were made before those of the 6-4 photoproducts. Regan, Carrier and Francis[26] note that pyrimidine dimers account for approximately 90% of the damage induced in DNA by UV light. The dimer is produced in significantly higher yields than the 6-4 photoproduct over the UV range and from chromatographic analysis of irradiated and acid hydrolysed DNA, Patrick[27] measured 6-4 photoproduct induction at 5% of the level of cyclobutane dimers. Later workers focussed attention on both the cis-syn dimer and the 6-4 photoproduct and it became apparent that the induction of the 6-4 lesion was sequence dependant, i.e. the 6-4 photoproducts occur at certain sites more often than cyclobutane dimers. Evidence supporting the mutagenicity of the 6-4 photoproduct was derived from experiments with the lacI gene of *E. coli* where hotspots for 6-4 product formation were also hotspots



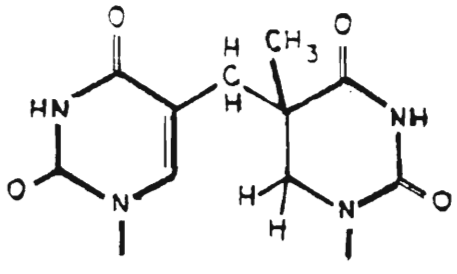
pyrimidine (6-4) pyrimidone



5,6-dihydro-5-hydroperoxy-6-hydroxothymine



5,6-dihydro-5,6-dihydroxythymine (thymine glycol)



5-thyminyl-5,6-dihydrothymine

Figure 1.5. Structures of selected UV-DNA photoproducts.

for induction of transition mutations[28]. This added weight to the body of evidence implicating the 6-4 photoproducts as being coparticipants with the cyclobutane pyrimidine dimer in mediating the lethal and mutagenic effects of UV light. Available data also suggests that the photoisomer of the 6-4 photoproduct (the Dewar pyrimidinone) is produced in significant amounts upon direct exposure of DNA to wavelengths between 280 nm and 360 nm and thus may also be involved in the mechanism of sunlight induced mutagenesis and skin cancer.

In vitro and *in vivo*, cyclobutane pyrimidine dimers and the 6-4 photoproduct are formed between two contiguous bases on the DNA strand under the influence of UV light. In the case of the cyclobutane dimer, photodimerization occurs via a [2+2] cycloaddition reaction of the pyrimidine bases across their C5-C6 double bonds. The most abundantly produced of these dimers is the thymine-thymine pyrimidine dimer, although reactions between cytosine-cytosine and thymine-cytosine bases do occur to smaller extents. As will be shown later, artificially introduced photosensitizers can be employed to enhance the production of one kind of dimer (e.g. thymine-thymine dimer) over the other pyrimidine dimers.

When free in solution, the pyrimidine bases can give rise to four isomers of the dimer (as shown in Figure 1.6.) for thymine. If these bases are held within the DNA helix, then (as mentioned above) stereochemical constraints imposed by the helix and the phosphate-deoxyribose backbone permit only one of the four possible stereoisomers to form and in native DNA, only the cis-syn isomer has been detected[29]. Photodimerization of adjacent pyrimidine bases along the DNA strand causes deleterious effects to the secondary and tertiary conformational structure of the DNA molecule. Ciarrocchi and Sutherland[30] showed that an unwinding angle of 8° accompanies photodimerization of thymine in DNA, this

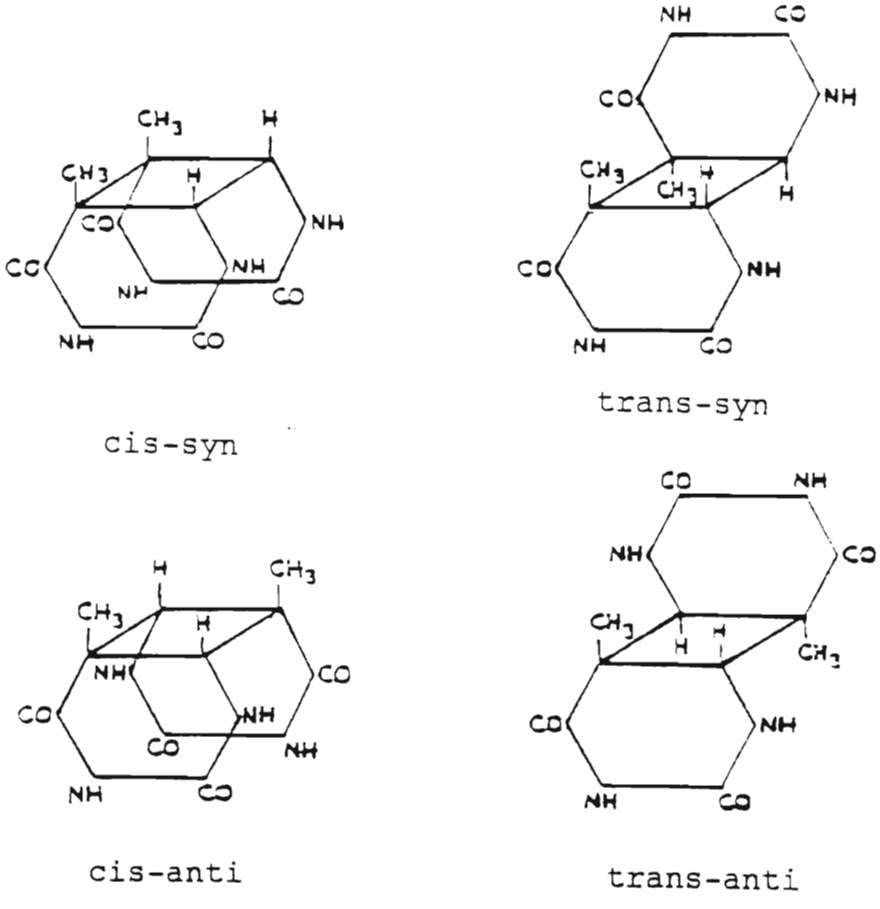


Figure 1.6. The four stereoisomers of the cyclobutane thymine dimer formed when an aqueous thymine solution is irradiated with UV light.

contributing to the measurable alteration of the tertiary structure of the DNA as a result of lesion formation. In support of these observations Pearlman et al.[31] calculated a bending angle of 27° after thymine dimerization in DNA. While these structural changes may be responsible in part for the harmful effects of these lesions in cellular systems, they may also represent recognition sites for repair enzymes. As will be discussed in Section 1.2., alterations to the tertiary structure of DNA as a result of dimer formation, is implicated as the initiating feature for antibody production to UV-irradiated DNA.

As a protective function, thickening of the epidermal layers and increased melanin production of the skin have developed as mechanisms in reducing the intensity of UV radiation reaching the basal layers where the carcinogenic process is thought to start. However, living cells have also developed a series of repair mechanisms which can remove UV-induced lesions. Setlow et al.[32] showed that cyclobutyl pyrimidine dimers in DNA are the substrate for the photoreactivating enzyme, PRE, and that this lesion alone is repaired by the enzyme[33]. In this light mediated photoreactivation pathway, the PRE enzyme binds to the lesion forming a complex which is stable in the absence of light[34]. Upon absorbing a photon of light in the 300 nm to 600 nm range, the enzyme converts the dimer to its two constituent monomers. In this manner, the fidelity of the particular DNA sequence is restored. Light independant repair processes also remove the damaged region of DNA[17,35,36]. The process involves excision of a dimer containing oligonucleotide and ligation of newly synthesized material (using the complementary DNA strand as a template) to the parental DNA. This rapid repair of active genes correlates with the resumption of RNA synthesis after UV irradiation[37] supporting the view that it is arguably the most important type of repair[35] of pyrimidine lesions due to its speed and precision. However,

post-replication repair is proposed by Zajdela and Latarjet[38], upholding the view that the excision repair pathway is inaccurate and that misrepair of the damage is responsible for the production of some neoplastic tumours. Several investigations have studied the rejoining of single stranded breaks in bacterial cells and in human fibroblasts. In the latter case, UVA induced single stranded breaks have a half life of 2 to 3 minutes at 37°C[39] which is similar to that for the repair of breaks induced by ionizing radiation[40]. Little, however, is known of the mechanism by which this repair occurs, or the mechanism by which repair of DNA-to-protein cross-links occurs.

Efficient removal of DNA lesions by cellular repair processes appears to be a crucial step in the prevention of tumour induction. In this regard, a number of human hereditary diseases such as xeroderma pigmentosum, Fanconi's anemia and ataxia telangiectasia have been shown to be unusual in their processing of damaged DNA components[41,42]. Xeroderma pigmentosum patients are unable to repair pyrimidine dimers and are highly susceptible to the development of skin cancers. This is due to a defect in a gene encoding the enzyme(s) that repairs pyrimidine dimers. People carrying the defect are a thousand times more likely to develop melanoma than people with functional copies of the gene[9]. However, DNA repair proficient revertant cells have been isolated from repair deficient cells, suggesting that a particular patient's condition may stem from a limited number of enzyme deficiencies. Another genetic disease concerned with an inability to repair damaged DNA is basal cell nevus syndrome where patients are pre-disposed to sunlight induced skin cancers. However, results indicate that DNA lesions and repair processes other than pyrimidine dimers are involved in the pathogenesis of this disease. Recently a human repair gene was cloned and when transfected into repair deficient

rodent cells the gene was able to correct the deficiency[43]. Reports such as this provide evidence of the mutagenicity of UV-induced lesions. If the lesions are not removed, then UV - induced mutations are important in initiating neoplastic events. Brash[44] has suggested that the frequency of mutation at a given point is more closely related to the local structure of DNA than to the photoproduct frequency at that point. This claim is substantiated by observations that while the presence of pyrimidine dimers may be a pre-requisite for UV mutagenesis, the actual mutagenic event can occur at a site some distance removed from the dimer[45].

Since the isolation of the cyclobutane pyrimidine dimer by Beukers and Berends in 1960[22], research on UV carcinogenesis has focused on a number of themes: attempts to define the relationship between UV exposure and induction of skin cancer in man and laboratory animals, description of the pathogenesis of the disease and investigations into the mechanism by which UV radiation causes cancerous tumours. To gain insight into the last mentioned line of research, workers have adopted a reductionist approach by investigating first the kinetics and mechanisms of dimerization of irradiated free pyrimidine base in aqueous solution. As mentioned previously, when free in solution, UV irradiation of thymine produces four stereoisomers of the cyclobutyl pyrimidine dimer. When studies were extended to examine photodimerization of DNA *in vitro*, only the cis-syn stereo isomer was detected. (This particular stereoisomer is also generated in frozen aqueous layers of irradiated thymine.) Irrespective of the environment of the pyrimidine base (free in solution or bound within the DNA helix), the dimerization reaction is photoreversible and a wavelength dependant photostationary state is achieved[22]. At wavelengths around 265 nm, the photoequilibrium favours dimerization, but at shorter wavelengths around 245 nm, the

equilibrium shifts to form thymine monomers. It is interesting to note that the thymine monomer absorbs UV light maximally at 265 nm, due to the conjugation of the C5-C6 double bond with the C4 carbonyl group. Since dimerization involves a [2+2] cycloaddition reaction across the C5-C6 double bonds of adjacent thymine moeities, it is logical to expect this characteristic absorbance to disappear upon dimerization (Figure 1.7.). If photoreversal is performed (by irradiating the thymine dimer with light of 245 nm) then the absorption at 265 nm will reappear due to thymine monomerization being favoured.

Photodimerization of thymine, uracil and cytosine in solution can be effected by direct irradiation using wavelengths less than 290 nm, particularly those wavelengths at 265 nm where the pyrimidine bases have a maximum UV absorbance, the energy of which is absorbed by the bases to initiate the dimerization process.

Photodimerization of pyrimidine bases is generally accepted to occur via a mechanism involving the triplet state of the specific pyrimidine base. The process is initiated by the thymine base interacting with a photon of energy, thereby converting the thymine base to its excited singlet state. Kinetic investigations reveal that the majority of the singlet states decay to the ground state by processes involving radiationless transitions. A small portion of the excited singlet states, however, undergo spin inversion (intersystem crossing) changing to a more stable and longer lived triplet state. It is this triplet thymine molecule which collides with a ground state thymine molecule to form the mutagenic thymine lesion. Bimolecular self-quenching and radiationless decay have been identified as processes which occur concomitantly with dimerization. However, this mechanism applies only to dilute thymine solutions (< 1 mM). If the thymine concentration exceeds this value, then aggregation phenomena become important due to

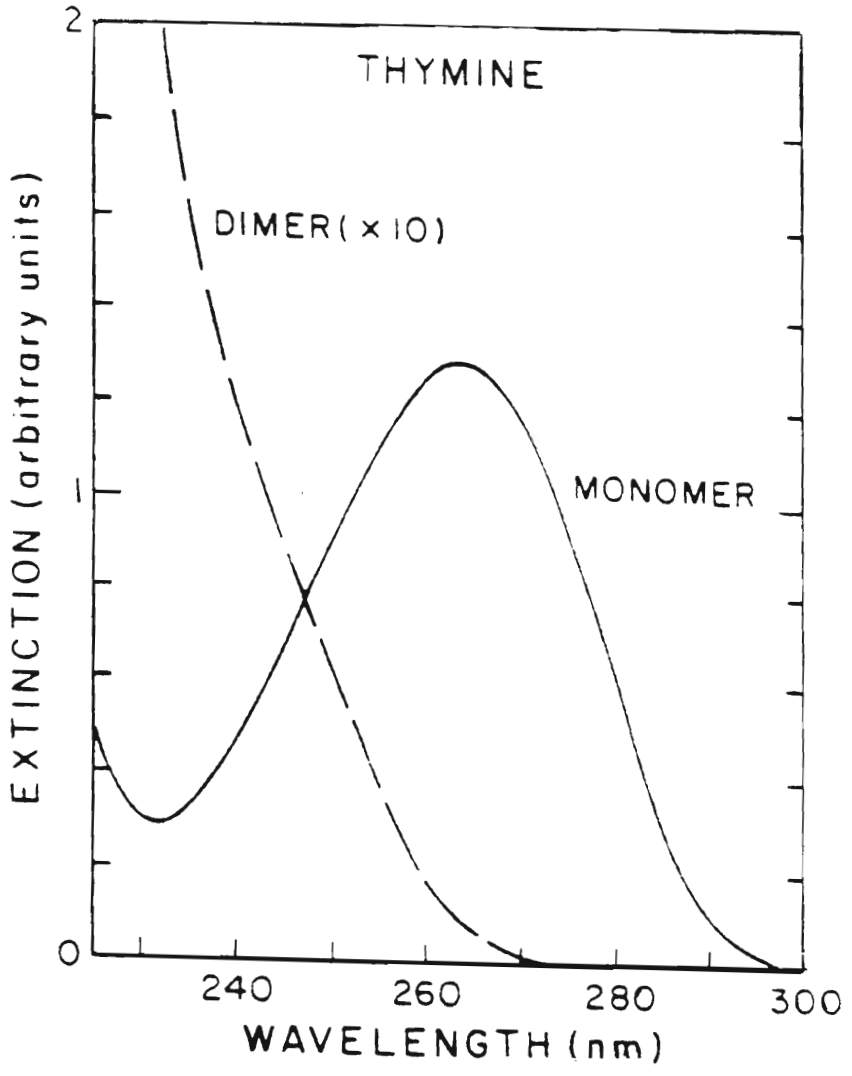


Figure 1.7. Characteristic UV absorption spectra of the thymine monomer and thymine dimer.

intermolecular hydrophobic interactions and dipole-induced-dipole interactions. Seeing that the thymine molecules are in close proximity (due to their aggregation), diffusion of isolated thymine molecules is not important and hence the dimerization process proceeds from population of the singlet state of the thymine.

This mechanism involving direct irradiation of the thymine base does not fully explain dimerization at wavelengths greater than 290 nm. At the UVA and UVB wavelengths (which do penetrate the stratospheric ozone layer), exogenous chemical photosensitizers (i.e. those that are added to the system) can be used to bring about indirect dimerization of pyrimidine bases in solution[46,47] as well as in DNA[49]. Ketones such as acetophenone, benzophenone and acetone have been investigated as possible photosensitizers of pyrimidine formation[46-49]. Many other photosensitizers have been identified, some of which act endogenously, but the majority are added manually to the cellular system. Included here are psoralen derivatives, riboflavins and the abovementioned ketones. Sunscreen constituents, however, also have photosensitizing properties apart from their function in absorbing UV radiation to reduce skin reddening during exposure to the sun. Examples of sunscreen components which have been shown to enhance thymine dimer production include para-aminobenzoic acid[50], disodium-2,2'-dihydroxy-4,4'-dimethoxybenzophenonesulphonicacid[51] and 2-phenylbenzimidazole-5-sulphonic acid[51]. (In contrast with the mutational potential of UV induced lesions whose production is accelerated in the presence of photosensitizers, it should be noted that selected exogenous chemical sensitizers find benefit when employed in photodynamic therapy. During treatment, a sensitizer is incorporated into an organism and when exposed to light of the appropriate wavelength for absorption by the sensitizer, oxygenated products harmful to cell function arise, eventually resulting in the destruction of (tumour)

tissue[52].)

In this work, the use of acetophenone was chosen as a photosensitizer for UV-irradiation of DNA (see later). It has been demonstrated that irradiation of DNA with 254 nm light yields several different photoproducts, but the use of acetophenone and acetone with 313 nm light gives a product distribution heavily favoured for cyclobutane pyrimidine dimers[48]. In particular, acetophenone photosensitization has been demonstrated to lead to the almost exclusive production of thymine photodimers in DNA[49] although thymine-cytosine dimers form to a small extent (approximately 5% of the reaction product[53]).

An explanation of the selective production of thymine dimers with acetophenone photosensitization is given as follows: at the molecular level, several orbitals are available for population of the nucleic acid bases (Figure 1.8.). The intense absorption band in DNA is primarily due to π-π^* transitions, although n-π^* transitions do occur due to nominally forbidden intersystem crossing mechanisms. However, photosensitizers are also able to populate the n-π^* states when irradiated with light of 313 nm in the presence of a pyrimidine base. Irradiation with this wavelength does not directly excite the DNA since absorption at 313 nm is negligible. Acetophenone has a lower-lying singlet state than any of the bases and absorbs extensively at this wavelength (Figure 1.9.). Due to an intersystem crossing efficiency of almost unity, every singlet acetophenone molecule is converted to its triplet state. This triplet state lies below the triplet level of cytosine, guanine and adenine, but above the triplet level of thymine. Hence population of the triplet level of thymine occurs by collisional processes with the triplet acetophenone. In DNA this results in the nearly exclusive production of the cis-syn thymine dimer.

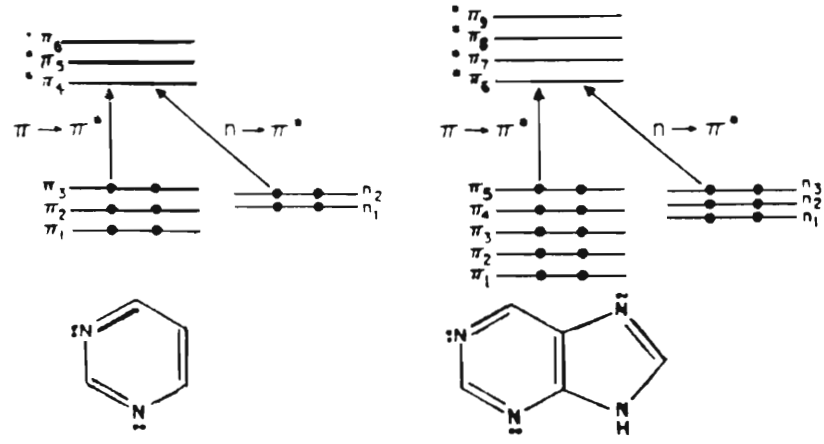


Figure 1.8. Diagram of energy levels available for light absorption in purines and pyrimidines[54].

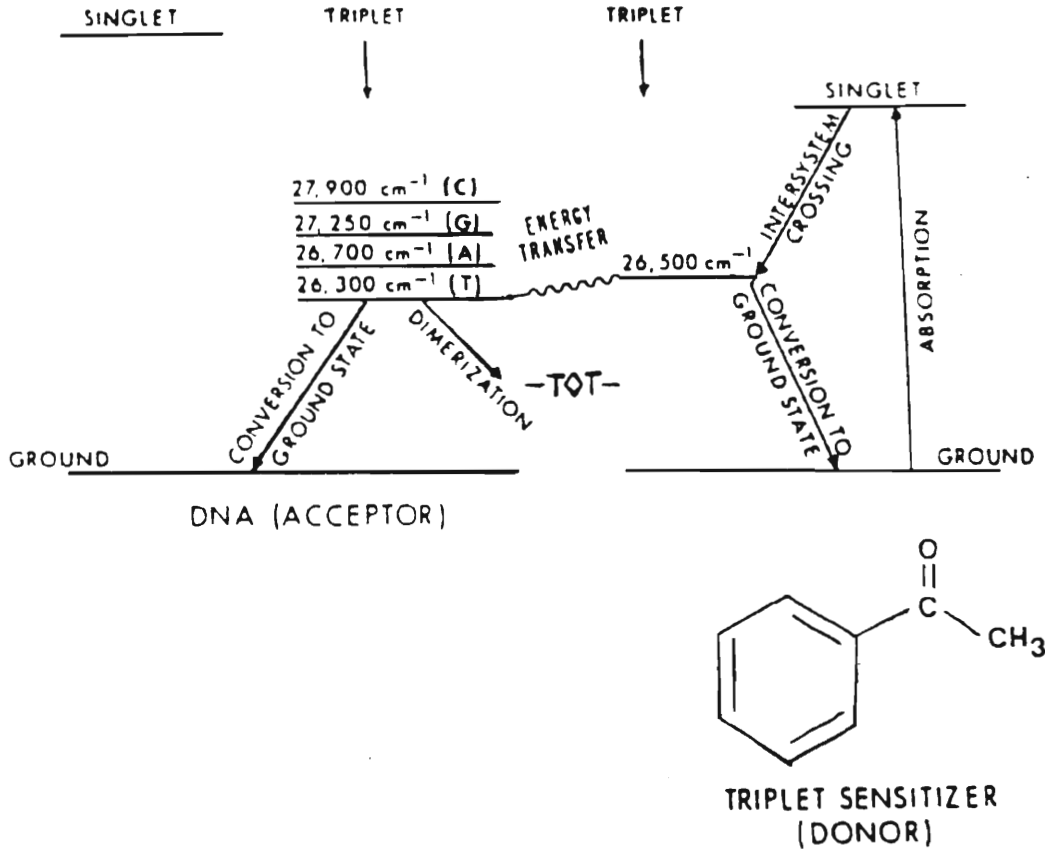


Figure 1.9. Schematic diagram of the main processes involved in acetophenone photosensitized dimerization of thymine in DNA[95].

The use of acetophenone as a sensitizer in this work is advantageous in that irradiation of DNA with acetophenone requires that only the cis-syn dimer (the main UV photoproduct) needs to be quantitated. From an immunological perspective, production of antibodies against UV-irradiated DNA in the presence of acetophenone implies that a single antibody population should, in theory, be generated against a cis-syn thymine dimer antigen, and not against any other UV photoproducts. This consideration is important in producing an antibody population of high specificity.

Previous work in this laboratory proposed a mechanism for acetophenone photodimerization in DNA[54]. Using a computer simulation of the reaction mechanism, Thomas[54] found good correlation with experimental data and this supported the validity of the mechanism. The proposed mechanism describes *in vitro* processes, and the work presented in this thesis provides a stepping stone towards quantitating thymine dimer production and elucidating a mechanism of photosensitized irradiation *in vivo*. To this end, Section 1.2 briefly reviews immunological perspectives of UV irradiation and in particular discusses aspects of antibody production against UV-irradiated DNA.

1.2 DNA PHOTOPRODUCT QUANTITATION - CHROMATOGRAPHY, RADIOLABELLING AND IMMUNOASSAY

The identification of the cis-syn cyclobutane dimer from frozen aqueous solutions of thymine by Beukers and Berends[22] in 1960 prompted researchers to investigate not only the mechanisms by which photoproduct formation occurs in irradiated systems, but also techniques whereby photodamage could be analysed and quantitated both *in vitro* and *in vivo*. Initially quantitation of the most abundantly produced lesion (the cis-syn thymine dimer) allowed correlations to be made with the mutagenic and cytotoxic

potential of this lesion, and thus implicated it in the reaction pathways that lead to UV-induced carcinogenesis.

In the early stages of investigations into DNA photochemistry, the most commonly used technique for analysing and separating photoproducts was descending paper chromatography. The technique is based on the relative solubilities of the photolesions in a given solvent system (e.g. isopropanol: water: ammonium hydroxide). However a 12 to 18 hour migration time was prohibitively time consuming and coupled with the problems of a lack of resolution of the cis-syn thymine dimer from the 6-4 photoproduct in the commonly used n-butanol: water: acetic acid solvent system[55], it was clearly evident that alternative techniques be employed. Although thin layer chromatography provided shorter resolution times (3 to 4 hours)[47,56], the procedure required that the photoproducts be detected by scintillation counting, the hazards of which will be highlighted later in this chapter.

Ion-exchange column chromatography was used to overcome the problems of co-resolution. Using this approach, Varghese and Patrick[57] resolved the cis-syn thymine dimer from the 6-4 photoproduct, and Weinblum and Johns[58] achieved resolution of the four thymine dimer stereoisomers from each other. With the advent of more sophisticated chromatographic techniques, both gas-liquid chromatography and high performance liquid chromatography (HPLC) were employed for photoproduct identification and quantitation. In spite of the advantages afforded by improved resolution and scale-up for ion-exchange, gas-liquid chromatography and HPLC, all of these techniques necessitate the degradation of DNA to the free base, nucleoside, mono- or oligonucleotide prior to analysis. Classical degradative techniques include alkaline hydrolysis, site selective enzymatic breakdown or hot acid hydrolysis of the UV-irradiated DNA (also referred to as UV-DNA) to cleave the

required photoproduct from the DNA strand.

Enzymatic hydrolysis exploits enzymes which sequentially degrade polydeoxyribonucleotides, yielding photoproducts which can then be analysed. However, chromatographic analysis may in some instances be complicated by the inability of nucleases to cleave the phosphodiester bond between a photoproduct and its adjacent base, thereby leaving an additional monomeric unit attached to each photoproduct. To ensure complete breakdown of the DNA to carbon, oxygen and carbon dioxide but simultaneously release stable photoproducts, the technique of hot acid hydrolysis is employed. Previous research in this laboratory[54] established a perchloric acid hydrolysis technique which breaks down the DNA to its constituent free bases and acid stable photoproducts. Analysis and quantitation of the relevant photoproduct is then performed using reverse phase HPLC with UV detection.

Whichever of the abovementioned techniques is employed for analysis and quantitation purposes, a distinct disadvantage is apparent since subjecting the DNA to harsh hydrolytic procedures does not allow the recovery of the intact irradiation substrate after analysis. A compounding problem is provided by observations that relatively large irradiation doses are necessary to induce HPLC detectable changes to the DNA molecule. These drawbacks led to the development of non-destructive analytical techniques for photoproduct analysis and quantitation, several of which allow the quantitation of low levels of photodamage in DNA without requiring radiolabelling of the relevant photoproduct. These investigative tools are largely enzymatic, immunological or molecular biological in nature. Included here is one of the most sensitive assays for identifying base damage which involves endonucleolytic incision at the site of the damage followed by analysis of the photoproducts produced in the DNA. In this manner

repair kinetics of pyrimidine dimers have been measured with an endonuclease isolated from either *Micrococcus luteus* or bacteriophage T4-infected cells. In this assay, measuring the number of chain breaks produced with alkaline sucrose sedimentation analysis allows base damage to be assessed[59,60]. Apart from identifying pyrimidine base damage, single stranded breaks at sites of 6-4 photodamage were induced by endonuclease activity and identified on gels using gradient sedimentation analysis.

As mentioned above, the sensitivity of these *in situ* techniques is greater than that obtained by chromatographic means and has allowed these biological techniques to be used for the detection of photodamage at lethal (10 J/m^2) and sub-lethal (0.5 J/m^2) UV fluences[162] where the production of cyclobutyl dimers is too low for quantitation by chromatographic means. Included in this group of highly sensitive non-destructive assays is radiolabelling (^3H or ^{125}I) of the damage. Lesions are quantified by scintillation counting of radioactivity but the technique carries with it all the associated hazards of working with radioactive species including the observation of base damage induced by the radiolabel[61,62] itself.

With the requirement that cis-syn thymine dimers in this work should be detectable at concentrations below those currently detectable by HPLC (i.e. $< 10^{-6}$ M) an immunological approach (i.e. the production of antibodies against DNA photoproducts) appeared to provide the necessary sensitivity requirements. It must be stressed that the sensitivity limits which can be achieved using immunological detection and quantitation are comparable with those achieved using radiolabelling. However, the latter technique requires sophisticated instrumentation and carries with it the abovementioned disadvantages of working with radioactive species. Environmental considerations are also beginning to militate against radioactive material

usage - chiefly because of the expense and difficulties associated with waste disposal.

Immunological detection and quantitation of UV-photoproducts requires that a laboratory animal be injected with an aliquot of the photoproduct of interest (the antigen). Because this UV-DNA antigen is recognised as "foreign" by the animal, a complex series of biochemical processes are initiated by the immune system, aimed at ridding the animal of the alien molecules. The end result of this mobilization process is the production of a proteinaceous antibody population with combining sites that are complementary to unique molecular features of the antigen stimulus. Through a combination of van der Waals forces, hydrogen bonding and electrostatic forces, the antibody population binds the foreign antigen molecules in reactions of extremely high affinity. In this way the antigen is neutralized and usually eliminated from the animal.

It is common for an organism to synthesize a family of antibodies that respond to multiple features of the antigen (i.e. phosphate-sugar backbone, short nucleotide sequences, cis-syn thymine dimer, 6-4 photoproduct). Thus a particular member of the antibody family will be directed against a specific structural unit, or epitope (e.g. cis-syn thymine dimer), of the antigen (Figure 1.10.). Each member of this family is produced by a single cell type or clone. This gives rise to the term "polyclonal antibody", where a wide variety of clones are involved in the production of antibodies targeted against multiple epitopes of the antigen. In contrast, monoclonal antibodies are produced in the laboratory from a single clone. Their great advantage lies in the fact that all antibodies bind to the antigen in the same way and with the same affinity. This has important applications if the antibodies are being used in analytical, clinical or preparative biochemistry. However,

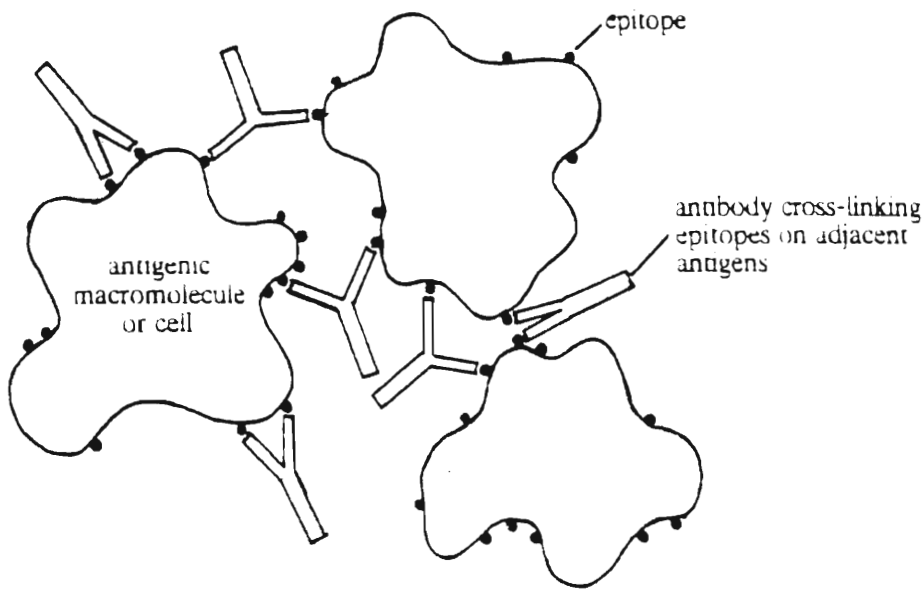


Figure 1.10. Antibody molecules binding to specific structural units (epitopes) of antigen molecules.

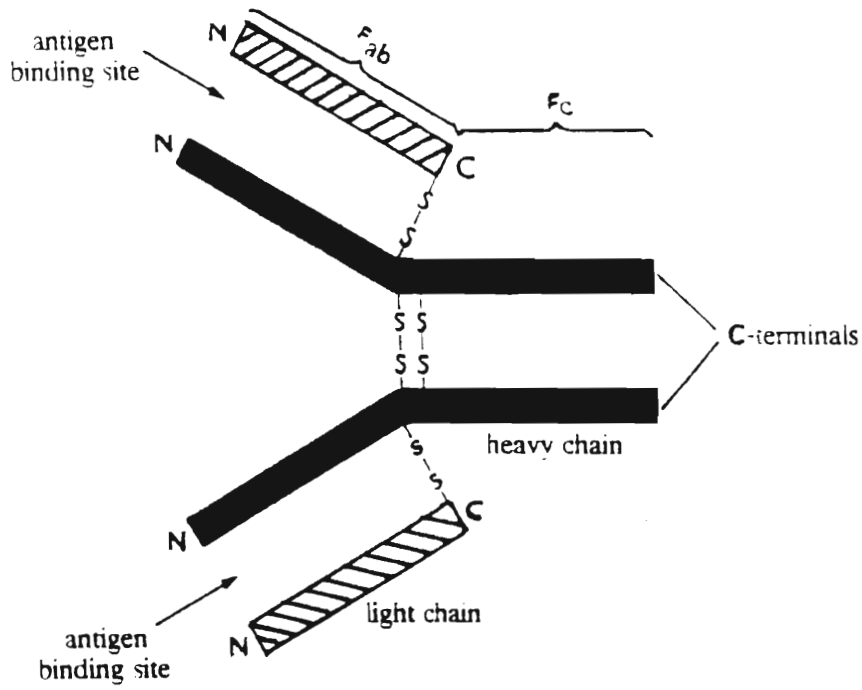


Figure 1.11. Schematic representation of an antibody molecule.

the hybridoma techniques that are essential for monoclonal antibody production require specialist knowledge and equipment. Hence in this work, polyclonal antibodies were produced in laboratory animals against UV-irradiated DNA.

Any antibody population whether generated against single or multiple antigen features is composed of a number of classes that differ from one another in chemical and physical properties. The antibody (or immunoglobulin) classes which are most commonly identified are Immunoglobulin G (IgG), Immunoglobulin M (IgM), Immunoglobulin A (IgA), Immunoglobulin E (IgE) and Immunoglobulin D (IgD). Of these, the IgG class is the most abundant in mammalian serum, comprising 75 to 80% of the total antibody complement[63]. The basic structural unit of all antibodies is composed of two light polypeptide chains and two heavy polypeptide chains joined by disulphide bridges (see Figure 1.11.). The molecular geometry of this basic structural unit resembles a "Y". In each arm of the Y (referred to as the F_{ab} region), the antibody binds the epitope of the antigen molecule, while the F_c region performs specific recognition functions. The antibody response resulting from exposure to antigenic molecules has certain well defined characteristics. After presentation of the antigen, intense protein synthesis occurs in lymphoid organs prior to assembly of antibody molecules. This primary immune response produces antibody populations which are detectable in the blood a week or two after exposure to, or immunization with, the antigen. This response does not persist and usually does not result in significant antigen production as can be seen in Figure 1.12. The situation is altered noticeably if a second immunization is administered. During this secondary response, antibody production is maintained at a high level, decreasing only slowly after a few months[63]. In the work presented in this thesis, antibody production was monitored in rabbits over the period of a year.

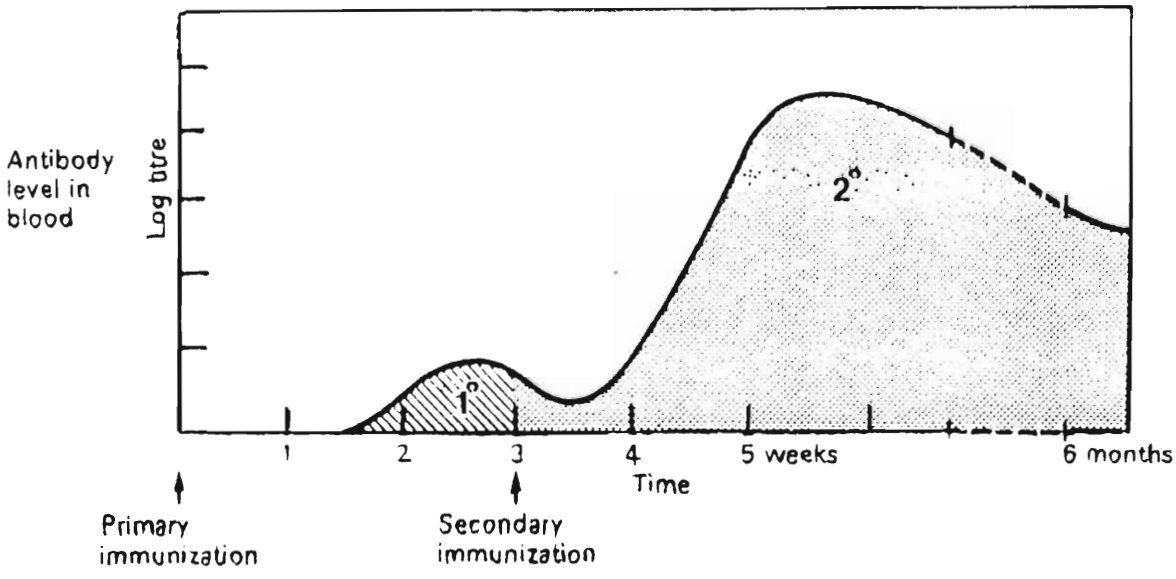


Figure 1.12. Typical antibody response curve, the antibodies produced after exposure of the animal to the antigen[63].

The question of antibody production against DNA or its constituent nucleotides, nucleosides or nucleic acid bases is an intriguing one. DNA is integral to every living cell and superficially it thus appears unlikely that the DNA molecule is immunogenic (i.e. antibodies can be generated against it). However, under specific experimental conditions (denatured DNA coupled to protein carrier molecules in the presence of "enhancers") and during the course of the autoimmune disease Systemic Lupus Erythmatosus, anti-DNA antibodies are elicited. (Aspects of DNA immunogenicity are discussed in Chapter 4.)

Early studies aimed at producing anti-DNA antibodies[64,65] were largely unsuccessful, primarily because the DNA (purified by 0.5% phenol extraction) contained appreciable amounts of protein and carbohydrate material against which antibodies were also produced. Alternative DNA antigenic substrates (wheat germ, calf thymus, herring roe) and improved purification techniques facilitated the production of anti-DNA antibodies with higher specificity. The most significant breakthrough, however, came with the description by Plescia et al.[66] of a technique which incorporated the protein carrier, methylated bovine serum albumin, with the DNA antigen prior to immunization (see Section 4.2.1.4). When the above technique was combined with the use of enhancers (adjuvants) antibodies were elicited against both single and double stranded oligonucleotides[67], synthetic polynucleotides[68] and RNA[69]. These antibodies cross reacted to different degrees with DNA from other sources and denatured DNA was shown to be the serologically active species.

The first production of antibodies directed against modified DNA components was demonstrated in 1966. Van Vunakis et al.[70] generated antibodies in rabbits to photo-oxidized DNA, while Levine et al.[71] pioneered antibody production against UV-irradiated and denatured DNA. This

initial demonstration was followed by several other reports[71-74] of anti-DNA antibody production and in most cases a detailed examination of the antigenic specificity of the resultant antibody was undertaken. The epitope or antigenic determinant against which antibody production is initiated is the cyclobutane pyrimidine dimer, as suggested by short wavelength reversal[71], enzymatic photoreactivation of the photoproducts[72,73] or by a correlation of the antigenic reactivity with expected and/or chromatographically measured repair kinetics of dimers[74]. Other studies demonstrated antibodies specific for a photoproduct of thymine[71,75], or a photoproduct of adjacent thymines[76] suggesting that thymine dimers were the preferred determinant as opposed to cytosine-thymine dimers. Furthermore, acetophenone photosensitization was used to prepare cis-syn thymine dimers in DNA, against which antibodies were generated in laboratory animals[77,78]. In this context it should be noted that although antibodies have also been raised against 6-4 photoproducts (mentioned previously), references to antibody production against other non-dimer photoproducts are scant and include only a reference to antibody production against thymine glycols[79].

In the majority of the abovementioned cases, polyclonal antibodies were produced in laboratory animals (usually rabbits). However, Strickland and Boyle[76] characterized a monoclonal antibody that was specific for thymine dimers in polynucleotide or oligonucleotide sequences of at least four bases long. Monoclonal antibody production has also been used extensively to characterize 6-4 photoproducts[58], and most recently a group of Japanese scientists produced monoclonal antibodies which recognized both thymine dimers and 6-4 photoproducts[80].

Irrespective of the nature of the antibody produced against UV-irradiated DNA, consensus is reached among investigators

that antibody production is initiated by antibody producing cells responding to conformational changes induced by dimerization, rather than the pyrimidine dimer itself[81]. There is also fair agreement that the binding sites on the UV-DNA encompass the backbone of one or both helical chains of the DNA over a span of about three to six nucleotides or base pairs[82]. These values are similar to the binding sites of antibodies that bind to undamaged DNA (about 5 nucleotides) and undamaged synthetic polynucleotides (4 to 6 nucleotides). In terms of the antibody combining site, which is estimated to be 24 to 36 Å in length, a segment of DNA 40 to 50 base pairs in length can allow bivalent high affinity binding by an IgG molecule[3] (Figure 1.13.). For a single pyrimidine dimer antigenic determinant, the observation that 3 to 6 nucleotides are necessary for antibody binding suggests that the presence of one or more adjacent unaltered bases on one or both sides of the dimer is necessary to provide stability to the antigen/antibody complex[74]. However, such flanking nucleotides probably do not function as antigenic determinants, as substition of different nucleotides into the flanking positions alters but does not eliminate, antigen binding.

Investigations into the nature of the antibody binding site, the antigenic determinants and the specificity of an antibody population have necessitated the development and establishment of analytical techniques to examine these phenomena. Shortly after methods for the production of anti-UV-DNA sera became widely available, sensitive immunoassays were reported for the detection of photoproducts in biological samples. The earliest of these made use of the immunological technique of complement fixation to indicate properties of the antibodies generated against UV-photoproducts. However, this assay was unable to detect damage caused by low levels of irradiation. Hence immunofluorescence (labelling the photoproduct, or antibody generated against the photoproduct with a fluorescent tag)

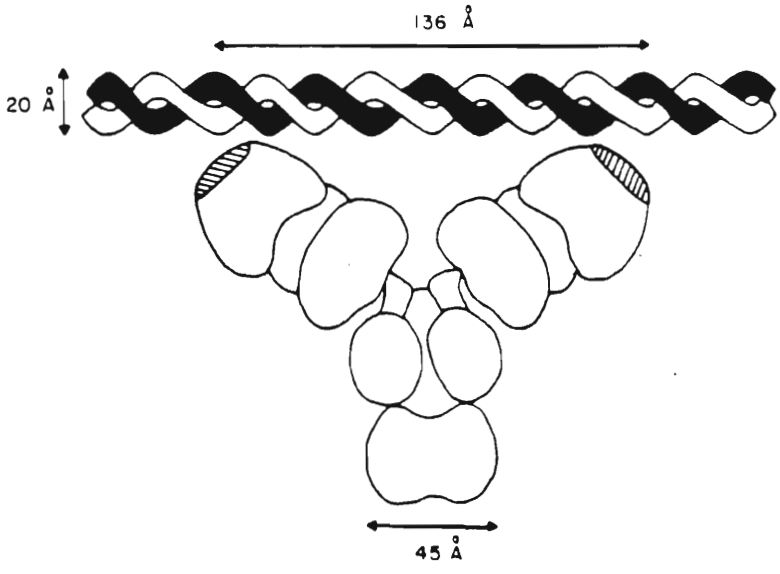


Figure 1.13. Size considerations allow an IgG antibody to bind a DNA segment of 40 to 50 base pairs with high affinity[3].

was developed and has been used extensively to observe the repair kinetics of irradiated cells and to detect dimers in the nuclei of human amnion cells irradiated with a dose of 30 J/m^2 [77]. Since immunofluorescence is not strictly quantitative and does not reveal lesions induced by UV fluences of less than 10 J/m^2, an alternative technique of radioimmunoassay was developed which permits the detection of photoproducts in intact DNA[74]. In this assay, usually ^{125}I is introduced into the DNA prior to irradiation and denaturation. Then the binding between [^{125}I]-UV-DNA and the anti-UV-irradiated serum is inhibited by small quantities of DNA irradiated with low doses of UV light. Scintillation counting of the resultant antigen/antibody complexes allow conclusions to be drawn concerning characterization and quantitation of photoproducts. The limits of sensitivity of this assay allowed the detection of photoproducts generated at UV fluences as low as 5 J/m^2.

More recently an enzyme linked immunoabsorbent assay (ELISA) was reported[82] to measure pyrimidine dimers in DNA. Exploiting the interactive and highly specific forces of immunological components: antigen, antibody and a secondary antibody specific for the primary antibody are immobilized in the wells of a microtitre plate. The secondary antibody is attached to an enzyme, the substrate of which is added to the immune complex and a colour reaction (catalysed by the enzyme) proceeds. The intensity of the reaction product allows quantitation of either the antigen or antibody to be made. Using the same principle, the immunoblotting assay has been described[83] which also immobilizes immune complexes on a support medium (usually a commercially synthesized membrane). After immobilization, the components of the immune complex can quantitated using radiolabelling[84] or by chemiluminescent techniques where the enzyme catalyses the production of light. Such an assay has been reported to detect UV lesions generated at sub-lethal (0.5 J/m^2) UV doses[162], in comparison with the

detection limit of 1 to 2 J/m^2 for the ELISA.

The brief discussion above has outlined available techniques used to quantitate antibodies generated against UV-DNA photoproducts which subsequently allow the photoproducts themselves to be quantitated. Because the development of an immunoassay method for the detection of thymine dimer lesions at low biological UV fluences is the central feature of the work presented here, more in-depth discussions concerning immunoassay techniques (and applications in quantitating thymine dimers), will be found in Section 5.1.

In the work presented here, it was imperative that low concentrations of thymine dimer be detected. Thus an immunological approach was adopted and antibodies to the UV-DNA antigen were raised in rabbits. Excluding the option of using a radioimmunoassay (because of the associated problems of dealing with radioactive material), an immunoblotting protocol was developed for thymine dimer detection and quantitation.

Once the methodology was optimized, the experimental procedures were used to determine thymine dimer yields at dimer levels undetectable by HPLC. Using the computer programme CAKE, these results were then used to test the mechanism proposed by Thomas[54] for the acetophenone photosensitized dimerization of thymine in DNA.

This thesis is divided into 6 chapters, the first of which has dealt with aspects of cyclobutane thymine dimer production in the context of DNA photochemistry (Section 1), and DNA photoproduct quantitation, examining specifically the approach of raising antibodies to UV-DNA photoproducts (Section 2). Chapter 2 deals with irradiation techniques for the production of the cis-syn thymine dimer in DNA, while Chapter 3 discusses photoproduct analysis and

quantitation by the technique of HPLC, currently used in these laboratories. Then the immunological aspects of DNA photochemistry will be considered - a discussion of the experimental aspects of the immunization techniques employed to raise anti UV-DNA antibodies (Chapter 4), and immunoassay for the quantitation of thymine dimer photolesions as used in this work (Chapter 5). In Chapter 6 the results obtained will be presented and discussed. Included here will be the application of immunoblotting in supporting the mechanism as proposed by Thomas[54] for photosensitized dimerization of thymine moeities in DNA.

CHAPTER 2 MATERIALS AND EQUIPMENT

The experiments reported in this thesis fall into four main categories. These are the experiments relating to the irradiation and HPLC techniques used in obtaining cis-syn thymine dimer yields (Sections 2.8.1 to 3.2.3), the experiments relating to the production of anti UV-DNA antibodies in rabbits (Sections 4.2.1 to 4.2.2), the experiments relating to the purification of the serum containing thymine dimer antibodies (Section 4.2.3) and experiments relating to the development and use of an immunoassay protocol for thymine dimer quantitation (Section 5.2.2). Listed below are the various chemicals and equipment used in the experiments carried out.

2.1 CHEMICALS FOR ACID HYDROLYSIS OF DNA

Perchloric Acid	(AR) SAARCHEM
Potassium Hydroxide	(AR) UNILAB
Polyethylene glycol	(LAB) SAARCHEM
Nitrogen Gas	High Purity

2.2 CHEMICALS FOR HPLC

Methanol	(HPLC) Waters
Perchloric Acid	(AR) SAARCHEM
Milli-Q Water*	

2.3 CHEMICALS FOR PHOTOLYSIS EXPERIMENTS

Calf Thymus DNA	Sigma
Thymine	(Sigma) Sigma
Guanine	(Sigma) Sigma
Cytosine	(Sigma) Sigma
Acetophenone	(LAB) SAARCHEM

2.4 CHEMICALS FOR ANTIBODY PRODUCTION

Calf Thymus DNA	Sigma
Potassium Chloride	(AR) BDH
Sodium Chloride	(AR) SAARCHEM
Potassium Dihydrogen Orthophosphate	(AR) BDH
di-Sodium Hydrogen Orthophosphate	(AR) SAARCHEM
Acetophenone	(LAB) SAARCHEM
Polyethylene glycol	(LAB) SAARCHEM
Methylated Bovine Serum Albumin	(Sigma) Sigma
Freunds Incomplete Adjuvant	(Sigma) Sigma

2.5 CHEMICALS FOR ANTIBODY PURIFICATION

Ammonium Sulphate	(AR) MERCK
NaOH	(LAB) BDH
Ammonia solution	(LAB) BDH
Rabbit IgG	(Sigma) Sigma
Tris-(Hydroxymethyl)amino-methane	(AR) SAARCHEM
Sodium Chloride	(AR) SAARCHEM
Glycine	(AR) BDH
Sodium Azide	(AR) BDH
Hydrochloric Acid	Prepared
Milli-Q Water*	

2.6 CHEMICALS FOR ANTIBODY DETECTION AND QUANTITATION

Methanol	(AR) BDH
Potassium Chloride	(AR) BDH
Sodium Chloride	(AR) SAARCHEM
Potassium Dihydrogen Orthophosphate	(AR) BDH
di-Sodium Hydrogen	

Orthophosphate	(AR) SAARCHEM
Tween-20	(AR) Riedel-de-Haën
Farmer's Pride Low Fat Powdered Milk	
Anti-rabbit Ig, horseradish peroxidase linked whole antibody (from donkey) (N930)	Amersham
ECL Western Blotting Detection System (RPN 2106)	Amersham
Phenisol Developer	Ilford
Hypam Fixer	Ilford
Milli-Q Water*	

2.7 EQUIPMENT FOR HPLC, AFFINITY CHROMATOGRAPHY AND IMMUNOBLOTTING

HBO 500W High Pressure Mercury Lamp (Osram)
Powerpack for HBO Lamp (Schreiber)
Blak-Ray J-221 Longwave UV Intensity Meter (UVP)
10 mm Pyrex Edge Filter
Irradiation cuvette
High Precision Micropipettes
- 100-1000 µl (Volac)
- 20-200 µl (Volac)
- 2-10 µl (Gilson)

600 Multisolvent Delivery System (Waters)
U6K Variable Injector (Waters)
990 Photodiode Array Detector (Waters)
990 Plotter (Waters)
APCII Personal Computer (NEC)
Microliter Gastight Syringe (10-100 µl) (Hamilton)
4000 Delta Prep: Preparative Chromatography

System (Waters)
7010 Rheodyne Injector (Waters)
486 Tunable Absorbance Detector (Waters)
745B Data Module (Waters)
Sterile Syringes (2.5 ml) (Terumo)
DMS 300 UV/Vis Spectrophotometer (Varian)
Suprasil Quartz 50 μl ultracuvette (Hellma)
Microliter Microcentrifuge (Hettich)
Immobilon-N Transfer Membrane (Millipore)
Fine, non-serated tweezers
Plastic boxes (10 x 10 x 8 cm^3)
Clingfilm
Shaking Waterbath (Protea)
Hyperfilm-ECL (18 x 24 cm^2) (Amersham)
Autoradiography Cassette (18 x 24 cm^2)
UMC 20 Ultrasonic Bath (Ultrasonic Manufacturing Company)
Number 3 Glass Vials (12 ml) (Polytop)
Sero-wel ELISA Plates (Sterilin)
Memsep 1000 Affinity Chromatography Cartridge (Millipore)
Ultracarb 5 ODS 30 HPLC Column (Phenomenex)
Model 25X Steam Sterilizer (Wisconsin All-American)
Millex HV 0.45 μm Filter Unit (Millipore)
Millex GV 0.22 μm Filter Unit (Millipore)
AE 200 Four Place Balance (Mettler)
1.5 ml Eppendorfs
Vacuum Filter Apparatus (Millipore)

Note*: Milli-Q Water refers to water that has been passed through the Millipore Milli-Q apparatus which consists of a series of ion-exchange and organic removal resins.

2.8 EXPERIMENTAL TECHNIQUES

2.8.1 IRRADIATION TECHNIQUES AND EQUIPMENT

Previous workers in this laboratory[50,54,85] have used a number of light trains for the irradiation of thymine (aqueous solution and frozen) and DNA (calf thymus and plasmid). Light sources used initially were mercury arc lamps (Hanau ST 40 and ST 75 medium pressure mercury lamps) which provide an ultraviolet output (line spectrum) in the 240 to 600 nm region. However, for the irradiation of *in vitro* DNA (aqueous solution of calf thymus DNA and pUC19 plasmid DNA), a high pressure mercury lamp was used by both Thomas[54] and Aliwell[50]. Since this work examines *in vitro* DNA irradiation, it was decided to follow these previous workers and use this high pressure mercury HBO 500W/2 lamp. The following section describes the use of this HBO lamp, its spectral characteristics, the irradiation cuvettes and filters used, and the irradiation techniques that were employed for the study of the photochemistry of DNA-acetophenone-phosphate buffered saline solutions.

2.8.1.1 Light Source for Quantitative Photolysis Experiments

Irradiation of *in vitro* DNA requires an intense light source. This is because dimerization of pyrimidine nucleotides is not a very efficient process when irradiation takes place at wavelengths greater than 290 nm and consequently quantitation of dimers is difficult. As mentioned above, a high pressure short arc mercury lamp (HBO 500W/2 supplied by Osram) was used (Figure 2.1.). The lamp consists of a high optical quality quartz glass discharge vessel containing mercury and a noble gas. The HBO produces an intense UV source with extensively broadened emission lines superimposed on a continuum (Figure 2.2.). Its special characteristics include

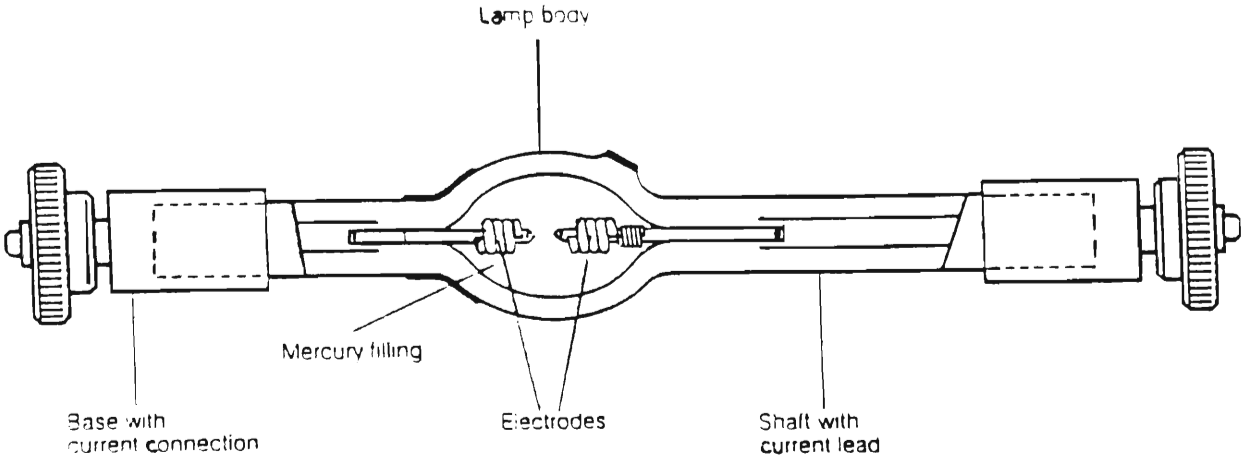


Figure 2.1. Osram HBO 500W/2 high pressure mercury arc lamp used for the irradiation of DNA.

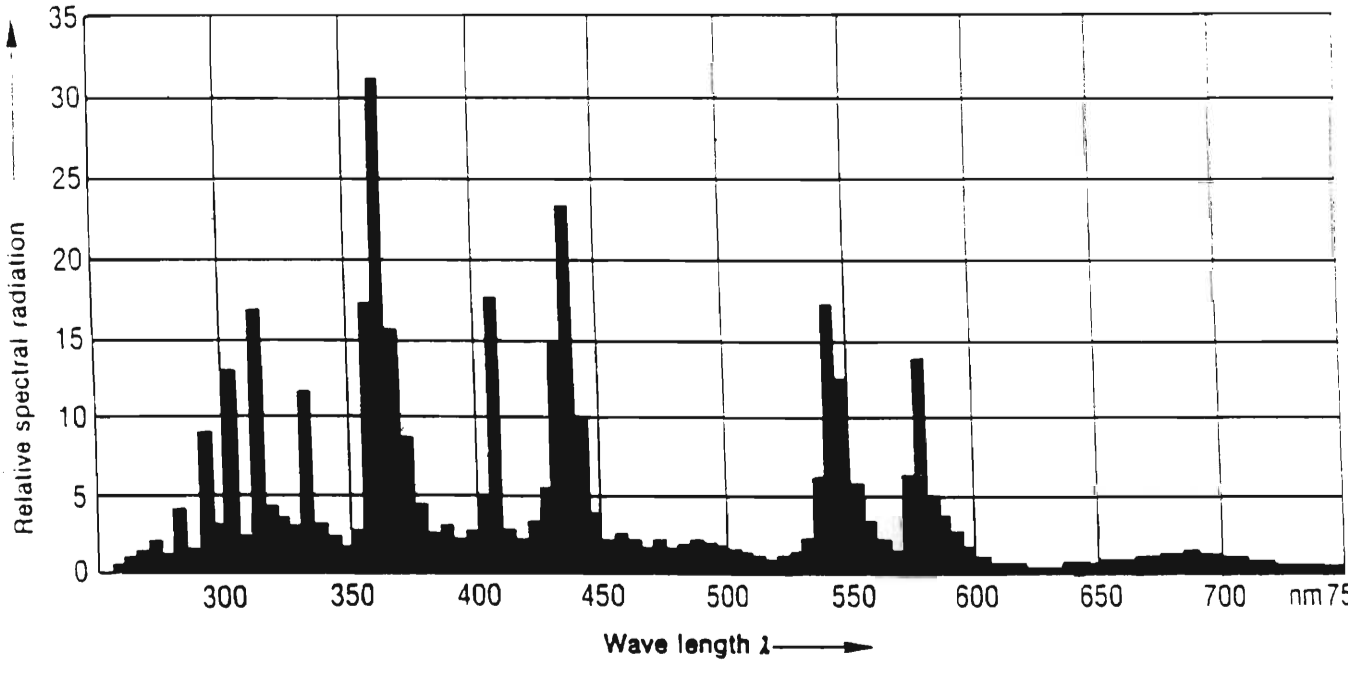


Figure 2.2. The relative spectral radiation distribution of the Osram HBO 500W/2 high pressure mercury arc lamp.

extremely high luminescence, high arc stability and a good luminous efficiency. Since that light of wavelength greater than 290 nm was used for the acetophenone photosensitized irradiation of DNA in this work, the HBO lamp in conjunction with a relevant Pyrex filter (see later), was a suitable UV light source.

It was important that the lamp be mounted free of mechanical stress and to allow for thermal expansion during operation. For best performance and high arc stability, the HBO lamp was operated in an upright position, but because of the dangers of UV radiation, overpressure during operation and glare, it was installed in a protective housing. The construction of this housing has been discussed in detail by previous members of the research group[54]. The lamp was housed in an insulated box constructed of mild steel through which a Schreiber DC power supply was attached to the lamp. A fan served to eliminate any ozone generated by the UV radiation. The UV radiation emitted from the lamp passed through a square aperture onto which the filter was attached and impinged on the cuvette containing the irradiation substrate. Externally, a bracket supported a shutter gate, filter holder and cuvette (Figure 2.3.).

Osram recommend a 400 hour lifetime for the HBO lamp and also that no quantitative radiations are performed until the lamp intensity is stabilized (usually after 3 to 5 hours). The lamp intensity was monitored *in situ* using a Blak-Ray J-221 Longwave photovoltaic UV intensity meter. The meter was used to measure the lamp intensity at intervals during the lamp lifetime, and thus allowed comparisons to be made between experiments performed at varying times of the lamp lifetime. Figure 2.4. illustrates the intensity variation of the HBO lamp that was used as a function of lamp hours.

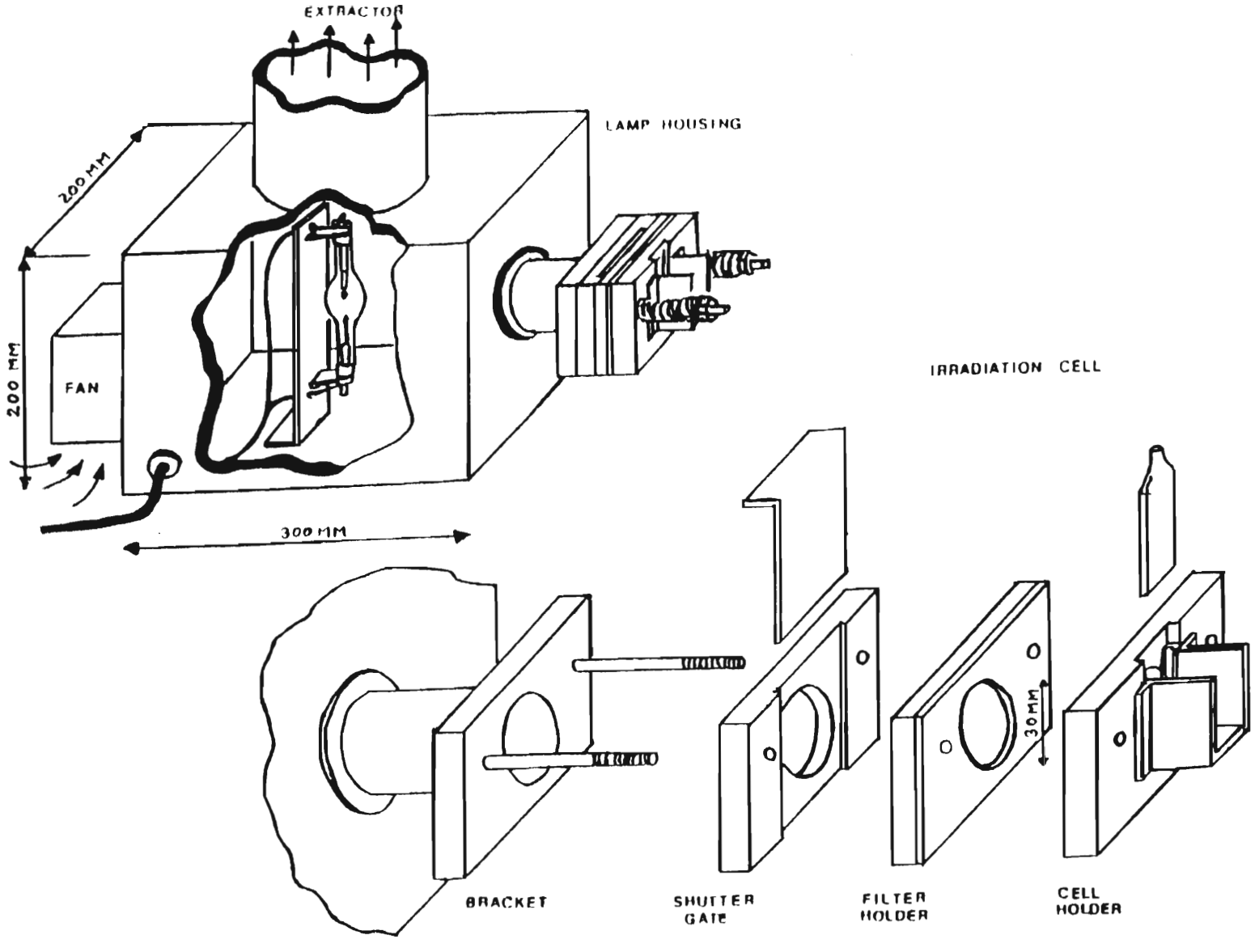


Figure 2.3. Housing used for the HBO lamp, illustrating the positioning of the irradiation cuvette in the irradiation train.

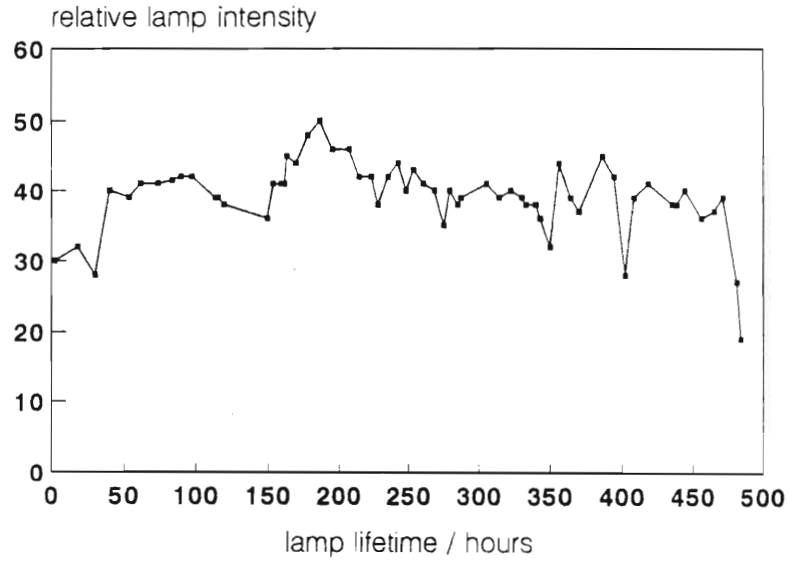


Figure 2.4. Relative intensity variation of the HBO lamp with lamp lifetime.

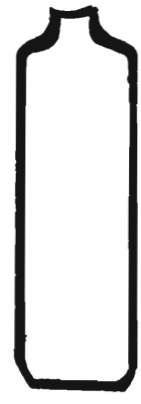


Figure 2.5. Short neck quartz irradiation cuvette.

2.8.1.2 Irradiation Techniques with the HBO Lamp

Quantitative irradiations were performed using a 1 mm thick short neck quartz cuvette (shown in Figure 2.5.). As a result, only small volumes of solution were irradiated at a time so that large concentrations of dimer could be produced within a reasonable irradiation period (10 hours or less).

Because DNA solutions are intrinsically viscous, the short neck cuvette has the advantage over a long neck cuvette of allowing more efficient removal of the irradiated solution for analysis. It was important to ensure that the cuvette was clean and dry before use in order that water adhering to the inner walls of the cuvette would not affect the concentration of DNA irradiated. To this end, the cuvette was immersed in a chromic acid solution before use, rinsed with copious amounts of Milli-Q water and then allowed to dry thoroughly.

As discussed in Section 1.1, a wavelength range of 290 to 320 nm was required for this work. Isolation of a specific wavelength or range of wavelengths from the UV source can be accomplished by using a monochromator, chemical filters or glass filters. Previous members of this research group have opted for Pyrex and narrow band filters and in this work, a 10 mm optically flat Pyrex filter was used for photosensitized irradiations of DNA.

Thomas[54] used a 1 mm optically flat Pyrex glass filter for photosensitized irradiations. This filter has a 40% transmittance at wavelengths less than 310 nm which allowed direct irradiation of the pyrimidine bases of DNA. Direct ($\langle$ 290 nm) irradiation of DNA causes the induction of thymine dimers as well as other pyrimidine (thymine-cytosine and cytosine-cytosine) dimers. On the other hand, indirect ($\rangle$ 290 nm) UV irradiation of DNA in the presence

of a photosensitizer induces the production of only the thymine dimer. In this work it was important that dimerization of only thymine bases was achieved because this served to reduce the number of antigenic determinants (molecules or groups of molecules against which antibodies are generated) exposed to the rabbit antibody producing cells. Thus, because only dimerization of thymine (and not cytosine) occurred, antibodies to thymine dimer alone should be produced.

However, to totally reduce direct dimerization of DNA, the transmission characteristics of a 4 mm Pyrex filter was determined. This was done by taping the filter over one aperture of the Varian DMS 300 double beam UV/Vis spectrophotometer and monitoring the percentage transmission over the 250 to 400 nm range. At 310 nm, the transmission was reduced from 40% to 35% which still allowed direct irradiation of the DNA. If the width of the filter was increased to 10 mm and the transmission characteristics determined as above, the percentage transmission at 310 nm was reduced to 6% (Figure 2.6.). In addition, the percentage transmission at 300 nm was calculated to be 3% and hence irradiation of DNA was largely eliminated at this wavelength. Thomas[54] however argues that the quantum yield for DNA dimerization at 310 nm is not significant and that direct dimerization of DNA causes negligible contributions to acetophenone dimer yields. Despite these observations, a 10 mm Pyrex filter was used because this latter filter allowed the photosensitized irradiation of DNA at wavelengths greater than 290 nm. This in turn reduced production of cytosine-cytosine and thymine-cytosine dimers hence ensuring antibody production against only the thymine dimer antigen.

In order to perform an irradiation, the 10 mm filter was positioned in the filter holder and all components of the external cradle bolted to the bracket. A 400 μl aliquot of

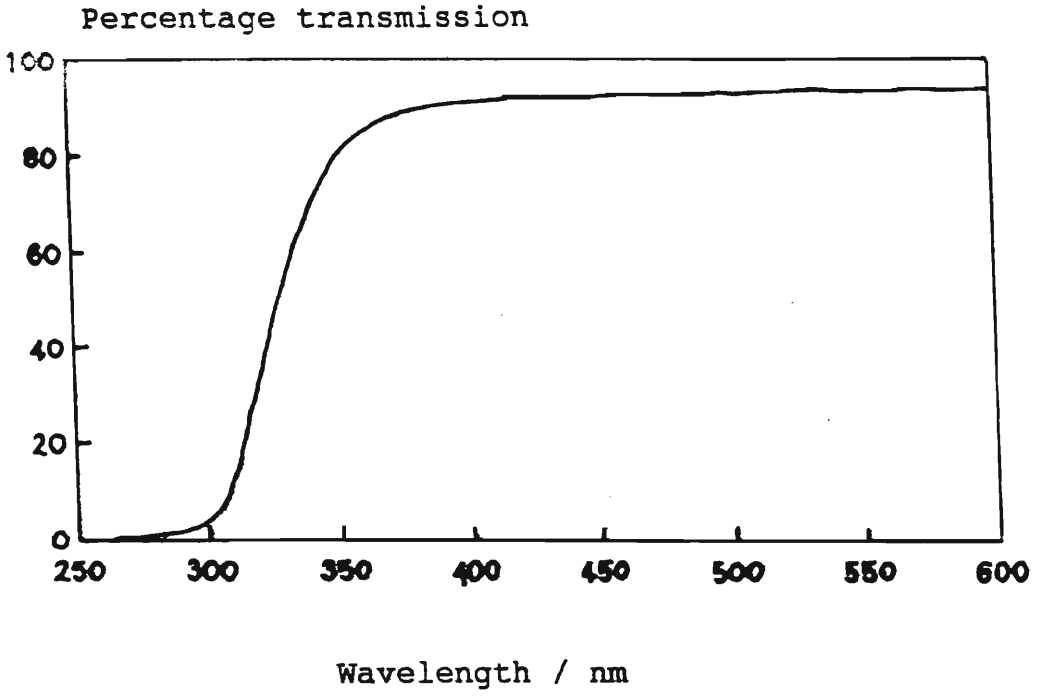


Figure 2.6. Transmission characteristics of the 10 mm Pyrex filter.

the DNA-acetophenone-PBS (phosphate buffered saline, Section 2.8.2.5) solution was transferred into a cuvette using a 100-1000 μl Volac micropipette. The neck of the cuvette was sealed using clingfilm to prevent evaporation of volatile acetophenone and PBS during irradiation. After a lamp warm-up of at least 15 minutes, the cuvette was positioned in the optical train.

2.8.1.3 Preparation of the Cis-Syn Thymine Dimer

In order to relate integrated thymine dimer peak areas obtained by HPLC to thymine dimer concentrations, a calibration curve of known dimer concentrations versus integrated peak areas was required. This necessitated the use of large quantities of cis-syn thymine dimer (the cyclobutane thymine dimer present in DNA). In addition, a solution of genuine (synthesized) cis-syn thymine dimer allowed peak identification during HPLC analysis via spiking or retention time measurements.

The method used in this work for the synthesis of cis-syn dimer was originally developed by Beukers and Berends[22], and involves irradiation of frozen solutions of thymine. An adaptation of the method has been used in this laboratory. Because of the positions of the thymine molecules relative to each other in a frozen ice matrix, dimerization of frozen thymine systems produces only the cis-syn stereoisomer of the thymine dimer[86]. At low thymine concentrations ($< 10^{-3}$ M), dimerization is thought to occur via the triplet state, while at high thymine concentrations ($> 10^{-3}$ M), aggregates or associated pairs of thymine molecules are thought to iniate dimerization via a singlet mechanism[87]. Although the exact value of the quantum yield for ice dimerization is debatable, the initial stages of dimerization are extremely efficient compared with aqueous solutions. (Values of between 1 and 2 have been estimated by Fuchtbauer and Mazur[88] in comparison with 0.65 for

splitting of thymine dimers in solution.) Thus significantly higher quantum yields for dimerization from ice irradiation can be compared with those observed for the irradiation of aqueous solutions. Hence large quantities of the cis-syn dimer can be prepared from frozen aqueous solutions of thymine.

The equipment used for the ice irradiations is shown in Figure 2.7. A Philips HP-T 400 W medium pressure mercury vapour lamp with its protective pyrex covering removed enabled wavelengths of less than 300 nm to impinge on the frozen thymine/ice system and allowed direct dimerization of the thymine monomers. An aluminium guard was fitted over the back of the lamp to provide support and protection if implosion occurred. The lamp was fitted into a black wooden box with an aluminium tray fitted to its base. Prior to irradiation, this tray was filled with finely crushed dry ice. A 2 x 10^{-2} M thymine solution (just below the thymine solubility in water) was prepared and sprayed onto the crushed ice, using a commercial insecticide sprayer to obtain fine and even coverage. A 1 mm thick layer of frozen thymine was formed. This layer was then irradiated using the Phillips HP-T lamp for three 5 minute intervals. It was important to keep a five minute interval between irradiations to prevent heat generated by the lamp from thawing the the frozen thymine layer and hence reducing the cis-syn thymine dimer quantum yield and allowing other isomers(cis-anti, trans-syn and trans-anti) to form. The irradiated thymine film was then removed using a spatula and allowed to thaw. The above irradiation procedure was repeated until approximately 500 ml of 2 x 10^{-2} M thymine solution had been irradiated.

Once irradiation was complete, the solution was heated to dissolve the precipitate and filtered hot in order that any particulate matter be removed. The filtrate was concentrated to 150 ml by evaporation on a hot plate after

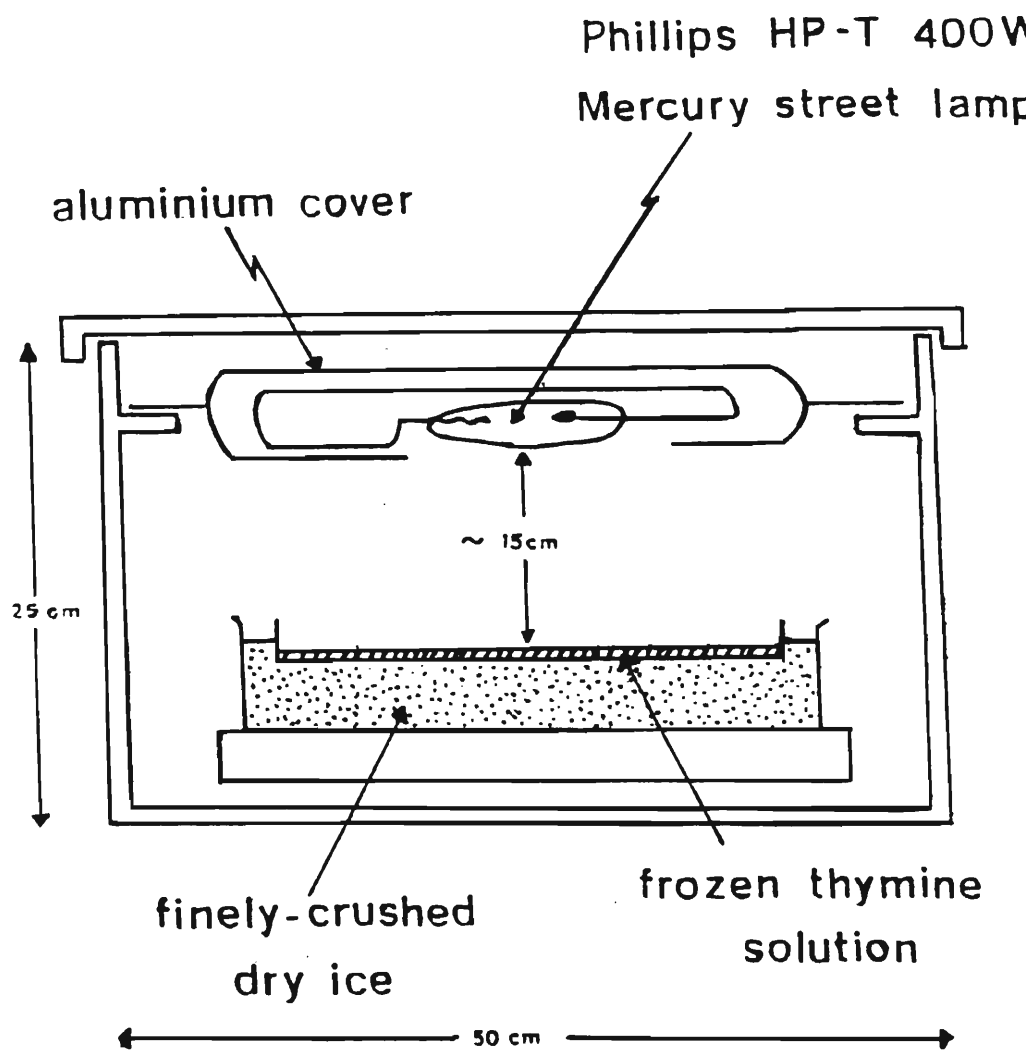


Figure 2.7. Apparatus used to generate cis-syn thymine dimer by UV-irradiation of frozen aqueous solutions of thymine.

which the solution was cooled and filtered to remove the dimer which had precipitated. Since small amounts of thymine monomer co-precipitate with the dimer, a purification stage is required. Purification involved washing the filtered solid with absolute ethanol in which thymine monomer is significantly more soluble than dimer. After a further washing stage using water, the dimer was then twice recrystallized from water at 80°C.

The purity of the cis-syn dimer produced was determined by UV spectroscopy and HPLC and found to be greater than 98%. The UV spectrum of a diluted dimer sample is shown in Figure 2.8.

The following section describes the preparation of DNA solutions for irradiation. First, the choice of calf thymus DNA as an irradiation substrate will be discussed together with the parameters of its purity and concentration which can be measured. Then, the use of acetophenone as a photosensitizer of only thymine dimerization will be discussed and this will be followed by a description of the manner in which DNA-acetophenone-PBS solutions were prepared.

2.8.2 PREPARATION OF DNA SOLUTIONS FOR IRRADIATION

A reductionist approach has been used by members of this research group in developing a kinetic model for the photosensitized irradiation of thymine. Starting with the irradiation of aqueous solutions of free thymine base, work was extended through studies of thymidine[110] to the irradiation of the more complex thymidylyl-3',5'-thymine[110,111] (TpT) system. This study was extended by examining the kinetics of irradiated calf thymus DNA and, then, finally to plasmid DNA[50]. Seeing that mechanisms have been proposed and simulated for all these thymine containing systems, it was logical to develop an

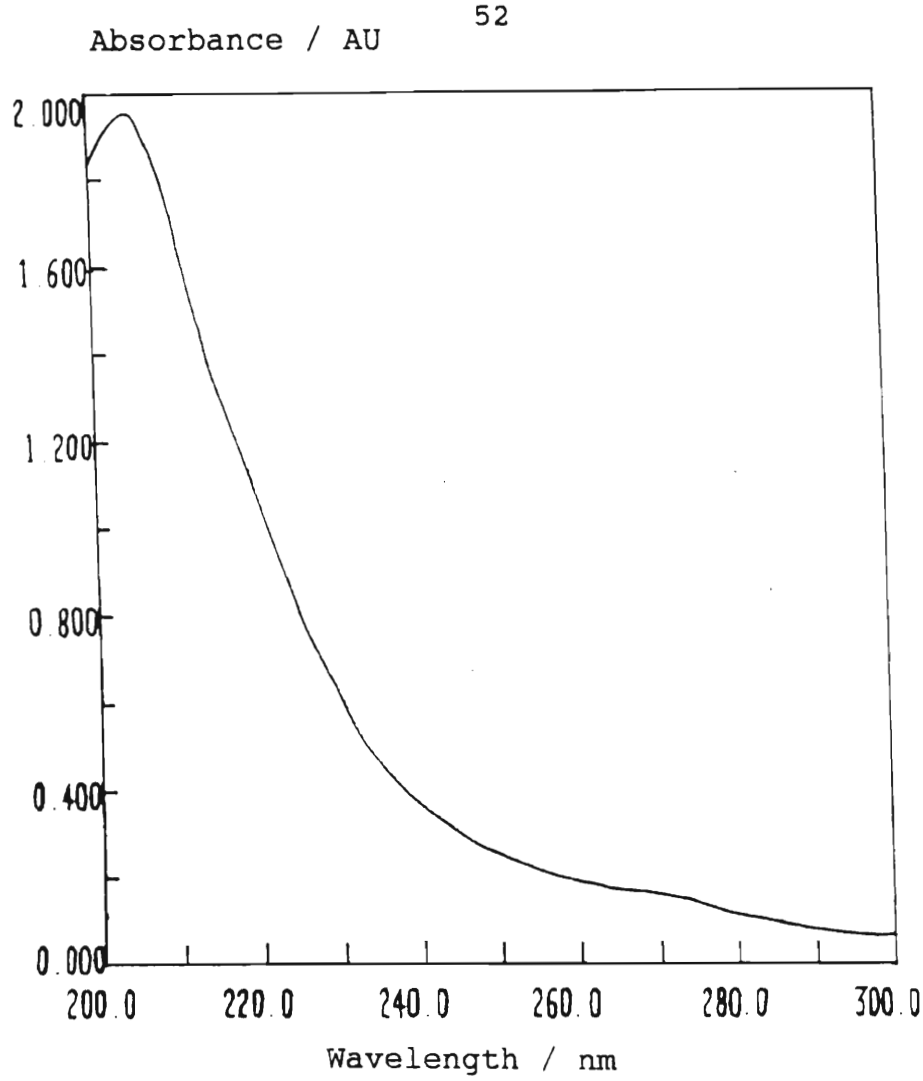


Figure 2.8. Characteristic absorption spectrum of the cis-syn thymine dimer.

immunoblotting routine using a DNA system, as opposed to an irradiated aqueous thymine system as the antigen in this immunoassay.

2.8.2.1 Use of Calf Thymus DNA

Calf thymus DNA was used by a previous member[54] of this research group and to facilitate mechanistic studies that were performed in this work (Section 6.7.1), irradiated calf thymus DNA was used as the antigen. Sigma Type 1 highly polymerized calf thymus (double stranded) deoxyribonucleic acid sodium salt was thus injected into laboratory rabbits. This DNA has the following base composition[89]:

Adenine: 29.0%
Guanine: 21.2%
Cytosine: 21.2%
Thymine: 28.5%.

As can be seen, Chargaff's Rules[90] of % Adenine = % Thymine and % Guanine = % Cytosine are obeyed. As will be discussed in Section 4.1.1, native DNA is not immunogenic, but if DNA is denatured (by heating or chemical means), large quantities of antibodies are generated against it.

DNA is always associated with histone proteins within the nucleus of the cell. In the Sigma DNA preparation, this protein had been removed to give a white fibrous preparation containing less than 3% protein. The Sigma analysis was performed using a standard biochemical assay for protein determination. It was important that the protein concentration be low, so that the number of antibodies generated against protein be minimized.

In addition to considering the protein content of the DNA, the examination of other parameters can also be investigated to characterize the DNA. These include:
a. The absorbance maximum (λ_{max} = 260 nm) and UV spectrum in

the 220 nm to 320 nm range.

b. Melting temperature at which 50% hyperchromism (see Section 4.2.1.3.) is reached.
c. Purity, as defined by A_{260} / A_{280} = 2.
d. Concentration (usually measured in mg/ml).

All of these parameters have been investigated in this work, or by Thomas[54], using a Varian DMS 300 double beam UV/Vis spectrophotometer. DNA samples were prepared in PBS (without the addition of acetophenone) as outlined in Section 2.8.2.5 and a dilution of this solution (0.1 mg/ml in PBS) was scanned over the 220 nm to 320 nm range against a PBS blank. A 0.1 mg/ml concentration was chosen for UV absorbance measurements, since at this DNA concentration the absorbance was within the optimum efficiency range of 0.5 to 1.5. In all cases, the resulting absorbance maximum was in the range 258.5 nm to 259.1 nm and had an absorbance of approximately 1.2. The absorption spectrum of a 0.1 mg/ml buffered DNA sample is shown in Figure 2.9. (λ_{max} = 259.1 nm).

The melting temperature (indicative of the source of the specific DNA) of a DNA solution was previously measured in this laboratory. Although the procedure is experimentally demanding, Thomas[54] determined the melting temperature of calf thymus DNA to be 87°C (in good agreement with reported values for DNA sodium salt from calf thymus DNA of the same ionic strength and pH[89]).

The purity of the DNA used in this work, as well as the measurements of the concentrations of the solutions used, will be discussed in Section 2.8.2.2 and Section 2.8.2.3 respectively.

2.8.2.2 Purity of DNA Samples

Although the DNA as supplied by Sigma had less than 3% protein, it was important to ascertain whether this would

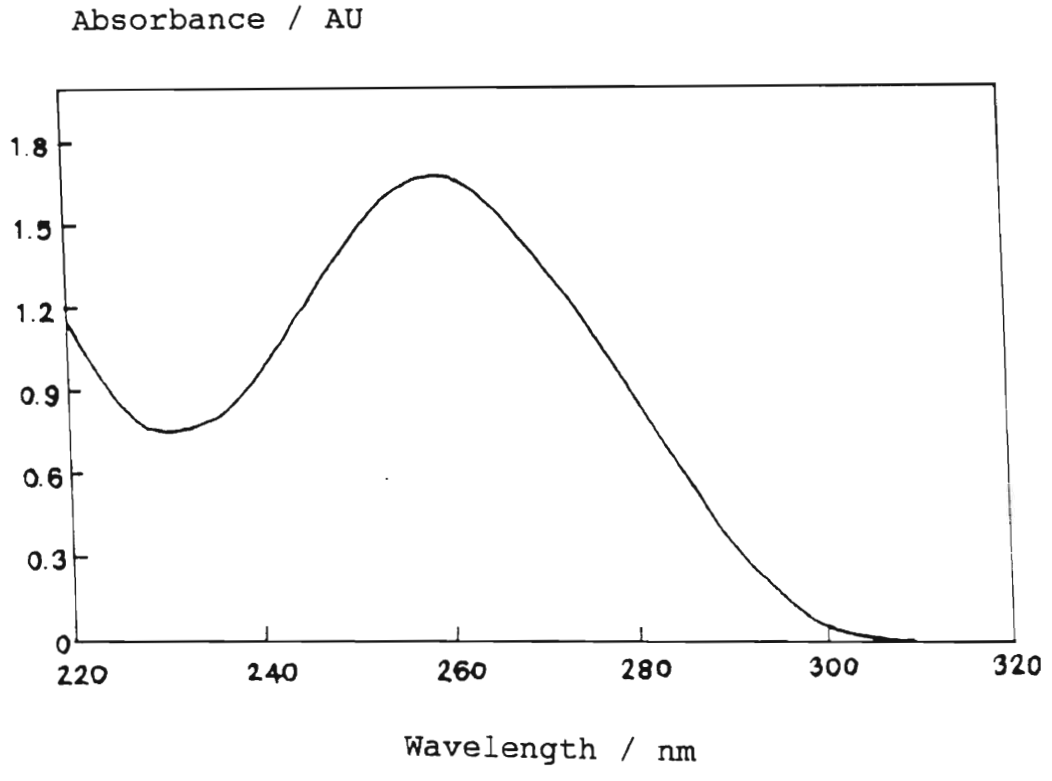


Figure 2.9. Absorption spectrum of a 0.1 mg/ml DNA sample.

interfere with photochemical experiments. The purity of a dilution of the stock DNA solution was determined by measuring the absorbance of an aliquot of this solution at 260 nm and 280 nm. The ratio A_{260} / A_{280} for pure DNA should be 2[91]. These measurements examine the absorbance of those protein impurities in the DNA which absorb at 280 nm. The absorbance is chiefly associated with the aromatic rings of the amino acids tryptophan and tyrosine (Section 4.2.3.1). In all cases, the ratio A_{260} / A_{280} for the calf thymus DNA yielded an answer of 1.85 or greater, thus indicating the absence of protein contaminant in the stock DNA solution.

2.8.2.3 Determination of DNA Concentrations

Thomas[54] determined the concentration of a DNA solution by weighing and UV absorbance measurements. The latter technique relied on determining the absorbance at 260 nm of an alkaline (pH 12) DNA preparation. Using the Beer-Lambert Law, $A = \epsilon cl$ and knowing the relevant extinction co-efficient, ϵ, which was specific for the system being used, the DNA concentration could be calculated. However, such investigations are fraught with error: primarily because quoted extinction co-efficients (or molar extinction co-efficients for proteins and nucleic acids) are strongly dependant on the buffer, pH and ionic strength of the solution under investigation. In addition these conditions are not always specified in the literature.

Taking into account the problems associated with the spectrophotometric determination of DNA concentrations, it seemed plausible to use weighing as a means of calculating DNA concentrations. The water content of nucleic acids is usually not known, so that measurement of concentration by weighing out a known amount of DNA, dissolving it in PBS and making up to volume could be in error. Sigma does, however, provide water and sodium percentages for each sample of DNA dispatched, and for the calf thymus DNA used

in this work, the DNA contained 9% sodium and 13.3% water.

25.55 mg of DNA was weighed out on a four place Mettler AE 200 balance. After correcting for the sodium and water present and making up to 25 ml in a volumetric flask the DNA concentration (by mass) was effectively reduced from 1.022 mg/ml to 0.7941 mg/ml. If a molar mass of 2×10^6 is used[54], the DNA solution is 3.970×10^{-10} M.

2.8.2.4 Use of Acetophenone as a Sensitizer

Irradiation of DNA with UV light less than 290 nm induces the formation of pyrimidine dimers via direct interaction between the light quanta and the target DNA. If light of greater than 290 nm is used for irradiation purposes, then a sensitizer must be added to promote dimer formation. (As was mentioned in Section 1.1, wavelengths greater than 290 nm do impinge on the earth's surface, whereas those less than 290 nm are filtered out due to the protective ozone layer.)

The photosensitizer of choice in this work is acetophenone (Figure 2.10.). The mode of action of acetophenone sensitization was explained in Section 1.1 and other members of this research group have investigated the kinetics of acetophenone photosensitized irradiation of aqueous thymine[92] and calf thymus DNA[54] in detail. Lamola and Yamane[93] first showed conclusively that irradiation of DNA at 313 nm in the presence of acetophenone results in the nearly exclusive production of thymine dimers in DNA.

The addition of acetophenone to irradiated DNA systems simplifies the photochemical studies of this system because only the cis-syn thymine dimer (which is the main UV photoproduct in irradiated DNA) is formed. This, in turn, aids chromatographic quantitation of photoproduct yields. Thus to facilitate antibody production against the main UV-

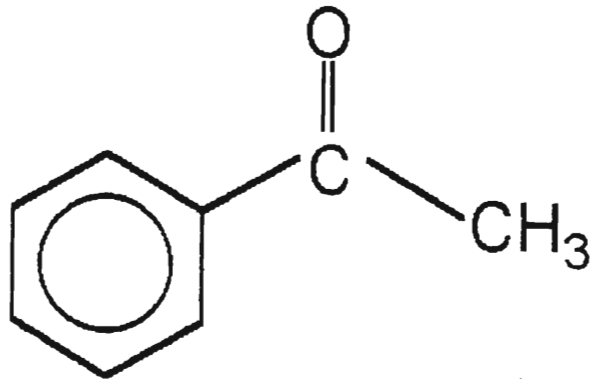


Figure 2.10. Structure of Acetophenone.

photoproduct (cis-syn thymine dimer), and minimize antibody production against any other pyrimidine dimers (cytosine-thymine or cytosine-cytosine dimers), acetophenone was chosen as the sensitizer.

Saarchem LAB grade acetophenone (1-phenylethanone; 120.15 g/mol) was used in this work. The UV spectrum of an aliquot of 4×10^{-2} M acetophenone in PBS is illustrated in Figure 2.11. The acetophenone does absorb at wavelengths greater than 290 nm and it can transfer its energy in a diffusion controlled reaction to the thymine bases. Rahn[94] has calculated the ϵ_{313} of acetophenone to be 105. In aqueous solution, the triplet lifetime of acetophenone is approximately 125 μs[94]. This is sufficiently long for diffusion controlled reaction with the thymine bases to occur.

The solubility of 2×10^{-2} M to 8×10^{-2} M acetophenone in PBS was determined by Thomas[54]. Results indicated that sensitizer concentrations greater than 4×10^{-2} M should not be exceeded because of extended time periods required for the sensitizer to dissolve. In addition, it is noted that thymine dimer yields for the same DNA sample irradiated for the same period, were significantly lower for a 4×10^{-2} M acetophenone solution as opposed to a 2×10^{-2} M acetophenone solution[95]. Although these observations were not observed by Thomas, they can possibly be explained in terms of acetophenone quenching of dimer yields. Hence, 2×10^{-2} M acetophenone was used for all photosensitized irradiations of DNA.

It is usual for DNA-photosensitizer solutions to be pre-bubbled with nitrogen for 15 minutes prior to irradiation. This serves to reduce dissolved oxygen to less than 10^{-6} M and hence excludes oxygen (and free radical) interference in kinetic modelling. However, Thomas[54] showed that nitrogen pre-bubbling was not necessary as it did not

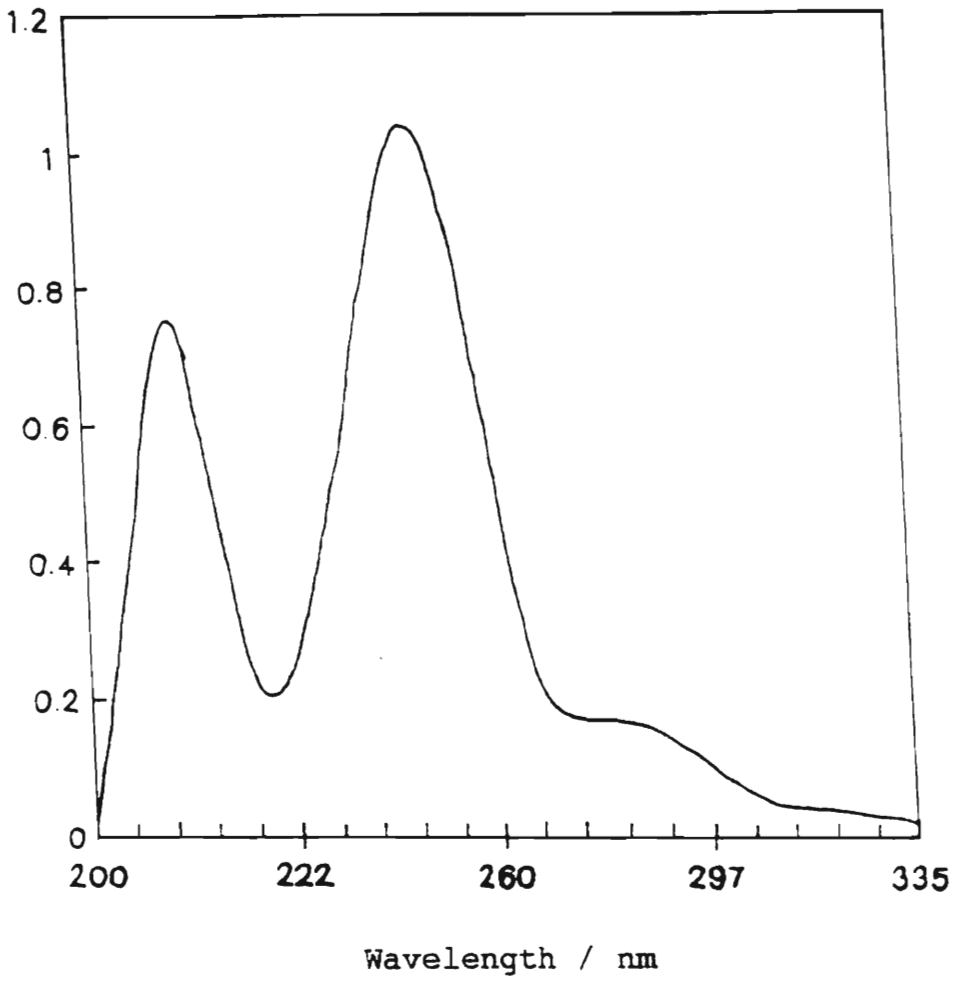


Figure 2.11. Absorption spectrum of acetophenone.

affect dimer yields under the experimental conditions employed. Thus in this work it was also excluded in DNA sample preparation prior to irradiation.

2.8.2.5 Preparation of DNA Solutions

As far as possible, sterile conditions were employed for sample preparation. Sterilization ensured that the immunogens injected into the laboratory animals were free of bacterial contamination and thus eliminated the animal mounting an immune response to them. In addition, sterilization prevented microbial degradation of the DNA as well as inhibiting destruction due to nucleases present on glassware.

Sterilization was routinely performed by autoclaving the glassware in a Model 25X Wisconsin All-American steam sterilizer for 20 minutes once a temperature of 250°C had been achieved within the sterilizer.

In order to reproduce physiological conditions, phosphate buffered saline (PBS) at a concentration of 0.15 M and pH 7.4 was used to dissolve the DNA. Solutions of PBS were prepared by weighing 8 g of NaCl, 0.12 g of KH_2PO_4, 0.20 g of KCl and 0.91 g of $NaHPO_4$. Analytical grade reagents were used in all cases. The salts were dissolved in a small volume (100 ml) of Milli-Q water and made up to 1 litre in a volumetric flask. The pH of the PBS buffer was tested using a Jenko portable pH meter calibrated against UniVAR pH 7.0 and 4.0 buffer solutions. The pH of the PBS buffer was between 7.3 and 7.4 in all cases. The PBS was filtered through Durapore 0.45 μm filters and sterilized as outlined above.

As mentioned in Section 2.8.2.4, acetophenone was used as the photosensitizer at a concentration of 2×10^{-2} M. The best means of dissolving the acetophenone was by sonication

for approximately 2 hours. Thus, the relevant volume of acetophenone (58 μl for 25.00 ml PBS) was transferred to a sterile beaker using a 20 to 200 μl Volac micropipette and 25 ml PBS added. The beaker was transferred to the sonicator and when acetophenone globules were no longer observed in solution, sonication was complete. Because acetophenone is volatile, the beaker was sealed with clingfilm or tinfoil during sonication.

The DNA was weighed on a four place analytical Mettler AE 200 balance. Because the DNA was particularly fibrous, tweezers were used for transferral of DNA during weighing. The DNA was then transferred to the PBS-acetophenone solution and allowed to dissolve by stirring on a magnetic stirrer in the coldroom (approximately -5°C) for about 2 days. The solution was transferred to a sterile "A" grade volumetric flask and made up to the mark with 0.15 M PBS-2×10^{-2} M acetophenone solution. Aliquots of this stock solution were transferred to sterile 1.5 ml plastic Eppendorf vials which were then frozen to inhibit microbial and nuclease activity. When required, the Eppendorf was brought to room temperature and the DNA solution (usually) irradiated as described in Section 2.8.1.2.

The following Chapter discusses the techniques whereby irradiated DNA samples are treated prior to analysis; in particular, the method of perchloric acid hydrolysis of DNA will be discussed prior to analysis by HPLC.

CHAPTER 3 HPLC TECHNIQUES

3.1 PHOTOPRODUCT ANALYSIS IN IRRADIATED DNA

The determination of the nature and concentration of photoproducts in a UV-irradiated DNA sample has been approached in two ways. The first technique relies on hydrolytic procedures which break down the DNA into its constituent free bases, nucleosides, mono- or oligonucleotides prior to analysis. This classical degradative technique involves hot acid hydrolysis, alkaline hydrolysis or site selective enzymatic breakdown of the DNA and is the means by which a photoproduct can be cleaved from DNA. Subsequent to degradation, photoproduct analysis has been performed via a number of chromatographic means. Initially, paper chromatography[96] and thin layer chromatography[97] were used, but these have been superseded by ion exchange chromatography[98] and gas liquid chromatography[99]. More recently HPLC techniques have been employed for photoproduct analysis and quantitation.

The second investigative tool for DNA photoproduct analysis involves non-destructive detection *in situ*. The techniques used are largely biological in nature and make use of enzymatic, immunological or molecular biological (e.g. use of restriction endonucleases) methodologies. Applications of these non-destructive techniques are found in Section 1.2. As was discussed in that section, the aim of the work presented here is the development of an immunoassay technique for the quantitation of thymine dimer. Although more sensitive than HPLC analysis, it utilizes methodologies applicable more to the realms of immunochemistry and biochemistry. Because the work reported here was performed in a physical chemistry laboratory, initially it seemed appropriate that a degradative technique should be chosen for analysis of DNA components

and hot acid hydrolysis had been used by Thomas[54] and Aliwell[50] as the mode of degradation of DNA. This technique will be described in the following section, and the HPLC routine which was adopted for quantitation of the photoproducts subsequent to degradation will be discussed in Section 3.2.

3.1.1 ACID HYDROLYSIS PROCEDURE

Hot acid hydrolysis using 9.2 M perchloric acid was used to degrade the UV-irradiated DNA. This was followed by reverse phase HPLC analysis of the hydrolysate in order that cis-syn thymine dimer yields be determined. The N1 glycosyl bond, joining the nitrogenous base to the deoxyribose sugar moeity, and the phosphodiester bond linking the sugar to the phosphate are broken during the hot acid hydrolysis. The deoxyribose sugar is completely degraded to carbon, carbon dioxide and oxygen. (The carbon is seen as a black deposit during hydrolysis.) Under the conditions employed (9.2 M perchloric acid; 100°C), the cyclobutyl dimer is stable and can subsequently be separated from other purine and pyrimidine bases by reverse phase HPLC.

The protocol which was adopted for DNA degradation and dimer analysis in irradiated DNA solutions was optimized in terms of perchloric acid concentration, temperature, duration of hydrolysis and perchloric acid:DNA ratios by Thomas[54]. The details of the final protocol are as follows:

a. The irradiated DNA sample was transferred to a Durham tube by inverting the quartz irradiation cuvette over the Durham tube until the contents were transferred.
b. The cuvette was rinsed with Milli-Q water and the washings added to the contents of the Durham tube.
c. The solution was evaporated to dryness under a gentle stream of nitrogen.
d. A 100 μl aliquot of 9.2 M perchloric acid was added to

the Durham tube and the tube sealed with clingfilm.

e. The tube was immersed in a polyethylene glycol bath thermostatted at 100°C for 1 hour and then cooled in ice for 15 minutes to halt hydrolysis.
f. The hydrolysed sample was transferred to a 1.5 ml Eppendorf vial, together with two 100 μl washings from the Durham tube.
g. The hydrolysate was neutralized with 10 M KOH. The final volume was 400 μl, the same as the volume originally irradiated. Because of the exothermic nature of the neutralization reaction, this was performed on ice.
h. The solution was centrifuged on a Hettich microcentrifuge (12 500 rpm) for 15 minutes.
i. A 30 μl aliquot of the supernatant was injected onto the HPLC using an Ultracarb 5 ODS 30 column with Milli-Q water eluant (pH 6) at a 0.7 ml/min flow rate.

3.2 PHOTOPRODUCT ANALYSIS BY HPLC

The chromatographic separation of nucleic acids and nucleic acid components (nucleotides, nucleosides and nitrogenous bases) was developed as a result of clinical investigations into defects of purine and pyrimidine metabolism [100] (causing such conditions as mental retardation, renal failure and toxemia). Separation of these nucleotides was originally performed using open column ion-exchange techniques, but the advent of High Performance Liquid Chromatography facilitated the isolation and quantitation of the nucleic acid constituents in biological fluids and tissues. Separations which originally took several hours using ion-exchange were dramatically shortened using HPLC. On-line detection systems meant that accurate characterization and quantitation of the solutes was achieved. In particular, octadecyl silica (ODS) C_{18} reverse columns were employed for the separation of nucleic acid constituents[101-106], while C_{18} columns have found application for the separation of photoproducts of

pyrimidines, nucleotides and nucleosides[107-109].

Reverse phase chromatography employs mobile phases which are more polar than the stationary phase (C_{18} ODS in this case). Hence, the more non-polar compounds, interact more efficiently with the relatively non-polar column packing and thus elute later than the polar sample components. Separation of these components is thus based on slight, though distinguishing, differences in polarity of the components themselves. The reverse phase packings which are used are faster to equilibrate than their normal phase counterparts, use less organic solvents (mobile phase) and have more versatile surface chemistries than bare (uncoated) silica used in the normal phase. In the work presented here, reverse phase chromatography has been used for the analytical separation of the components (nitrogenous bases and photoproducts) of UV-irradiated DNA.

3.2.1 HPLC EQUIPMENT AND OPERATION

A Waters 600 Multisolvent delivery system was used for this work. The system consisted of two 225 μl pumpheads, a U6K injector, a Waters 990 photodiode array detector, a NEC APC II personal computer and a Waters 990 plotter. Seeing that the operation of this equipment has been dicsussed in detail by other members of this research group[110,111], only a brief resume will be given here.

The multisolvent delivery system consisting of the fluid related unit and the electronics system, generates gradients using an interactive, microprocessor-based liquid chromatography system. This allows complex gradient programmes and clean up procedures to be run. The pump heads have a 225 μl volume, making this instrument suitable for semi-preparative and analytical work. The photodiode array detector consists of 512 photodiodes which provide a continuous UV/Vis monitoring of the sample from 190 to 800

nm. In this way, a full spectrum of the sample is provided with recall features allowing similarities and differences between chromatograms to be made. This computerized storage of data allows spectral and chromatographic overlays, contour plots and 3-D displays (absorbance versus wavelength versus time) to be generated. This latter feature is illustrated in Figure 3.1. Sample analysis and peak identification is thus greatly aided.

To increase column lifetime, the Ultracarb 5 ODS 30 column which was used in this work was washed and stored in HPLC grade methanol. To reduce contamination of the column by particulate matter or bacterial sources, all glassware was washed using chromic acid and the Milli-Q water used as the eluant in this work was collected just prior to use. A disposable Waters Guard-Pak μ-Bondapak C_{18} precolumn insert was included between the solvent delivery system and the column. The solvents were filtered through 0.45 μm Durapore filters. Removal of particulate matter protects sensitive equipment (optical cell, pumps, column etc.) within the HPLC. Filtration of the solvents also served to degas them. It was only necessary to filter the solvents prior to use. The solvents were subsequently stored in sealed bottles while the HPLC was operational. If the multisolvent delivery system was being used to generate gradients, then degassing was performed by purging the solvents with helium prior to and during use. Injections of samples onto the HPLC were made with a Hamilton airtight 100 μl syringe. The precision of injection was 1.0% as determined by multiple injections of a standard solution.

3.2.2 OPTIMUM CONDITIONS FOR CIS-SYN THYMINE DIMER ANALYSIS

An Ultracarb 5 ODS 30 analytical column has been used by members of this research group[50,54] for the separation, identification and quantitation of photoproducts generated

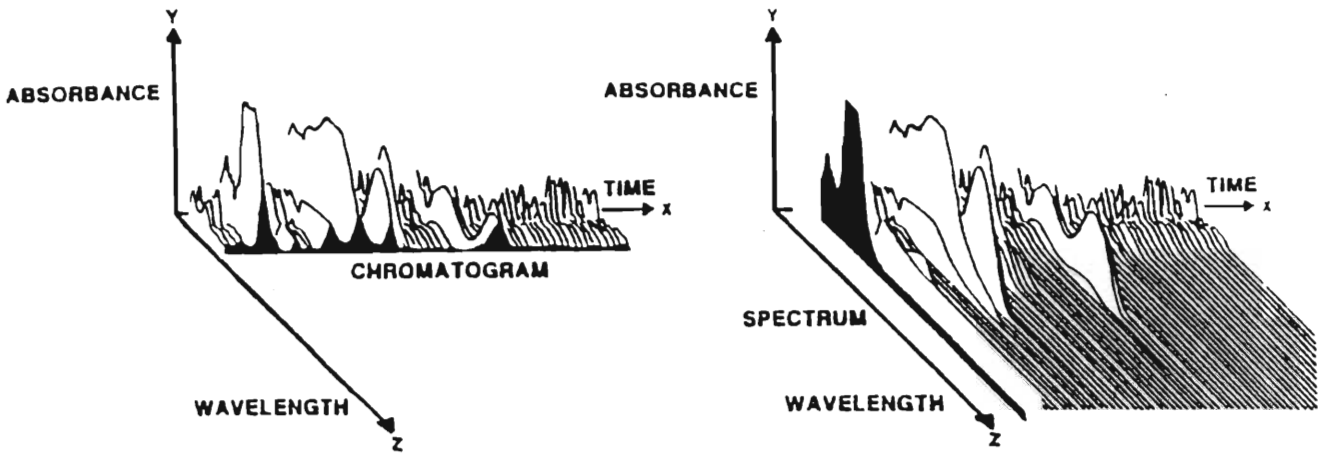


Figure 3.1. 3-D spectral and chromatographic data which is generated by the Waters 990 multiple photodiode array detector.

by the irradiation of DNA with selected photosensitizers and sunscreen constituents. This column was chosen over a range of similar C_{18} octadecyl silane columns for separation and identification purposes because of its superior resolution abilities in separating DNA components.

In addition, Aliwell[50] performed a series of column tests on conventional ODS as well as the newer Ultracarb columns. The chromatographic parameters of resolution (R), selectivity (α), capacity factor (κ) and efficiency (N) for the optimum separation of a 1 x 10^{-2} M thymine solution from a 2 x 10^{-2} M cis-syn thymine dimer solution were determined. Results indicated that Ultracarb columns outperformed the ODS columns, and that the semi-preparative columns were superior to analytical columns. This latter feature was not important for the work presented here as only analytical separations were employed, but these results illustrate the use of Ultracarb columns for the separation of nucleic acid nitrogenous bases and photoproducts.

Ultracarb 5 ODS columns are 250 mm in length with an internal diameter of 4.6 mm. A stainless steel casing houses the C_{18} packing material on silica gel. Particle size is 5 μm on average and all residual silanols are end capped. The Ultracarb 5 ODS 30 has 31% carbon loading, while its counterpart, the 5 ODS 20 has a loading of only 22%. The former column with its higher non-polar carbon component provides slightly enhanced separation for reverse phase chromatography.

As a starting point, the Ultracarb 5 ODS 30 was employed using Milli-Q water (pH 3) as the eluant at a flow rate of 0.7 ml/min. A 30 μl aliquot of the hydrolysed DNA sample was injected into the HPLC. The resulting chromatogram is illustrated in Figure 3.2. Identification of the various pyrimidine and purine bases and photoproducts present was performed by spiking the hydrolysate with an aliquot (5 μl)

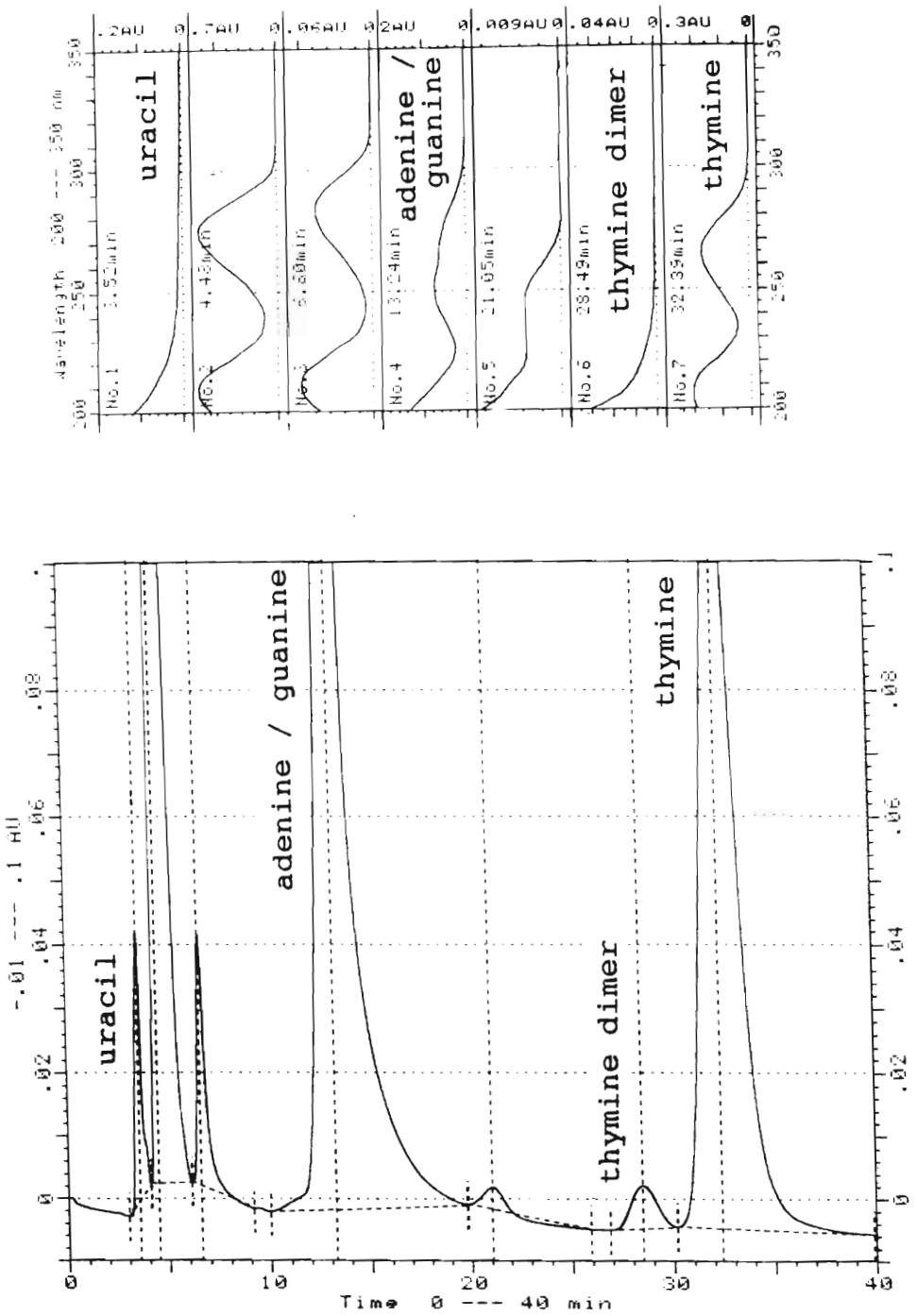
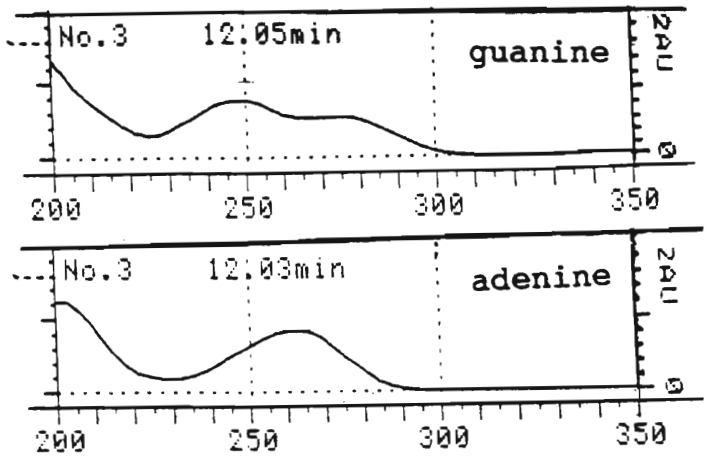


Figure 3.2. HPLC chromatogram showing separation of UV-irradiated DNA hydrolysate with a Milli-Q water (pH 3) mobile phase.

of the relevant compound, as well as by injecting an aliquot of a solution of the pure compound onto the HPLC and noting the retention time. It was imperative that the eluant and flow rate parameters used for the column be kept constant during the identification stages. In this manner, the cis-syn thymine dimer was identified as that component which eluted at 28.5 minutes, while the thymine monomer was identified by its retention time and UV spectrum to elute slightly later than the dimer at 32.4 minutes (monomer is more polar than dimer).

The guanine moeity eluted at 12.05 minutes when a partially soluble guanine solution was injected into the HPLC. Adenine was found to elute at 12.03 minutes. Comparison of the UV spectra with those obtained by Voet et al.[112] confirmed that these two components co-elute. Figure 3.3. illustrates the retention times of the pure guanine and adenine bases, together with their respective UV spectra. The cytosine base itself cannot be identified on the chromatogram because this base deaminates to uracil during hot acid hydrolysis. Spiking with a uracil solution and noting the retention time confirmed that this component eluted at 3.70 minutes with the salt peaks under the abovementioned conditions of eluant and flow rate.

Nucleosides as nitrogenous bases are relatively weak bases. Below pH 4.15 and 3.2 respectively, guanine and adenine will be protonated, while extremely low pH values (less than -3)[113] are needed to induce protonation of both thymine and uracil. Thus at pH 7, none of these bases should be protonated. Hence, to simplify solvent preparation and improve chromatographic separation of the purine and pyrimidine bases and photoproducts, Milli-Q water (pH 6) at a 0.7 ml/min flow rate was used as the eluant. The resulting chromatogram is seen in Figure 3.4. Improved resolution of the thymine monomer from the dimer is evident and hence this latter chromatographic regime



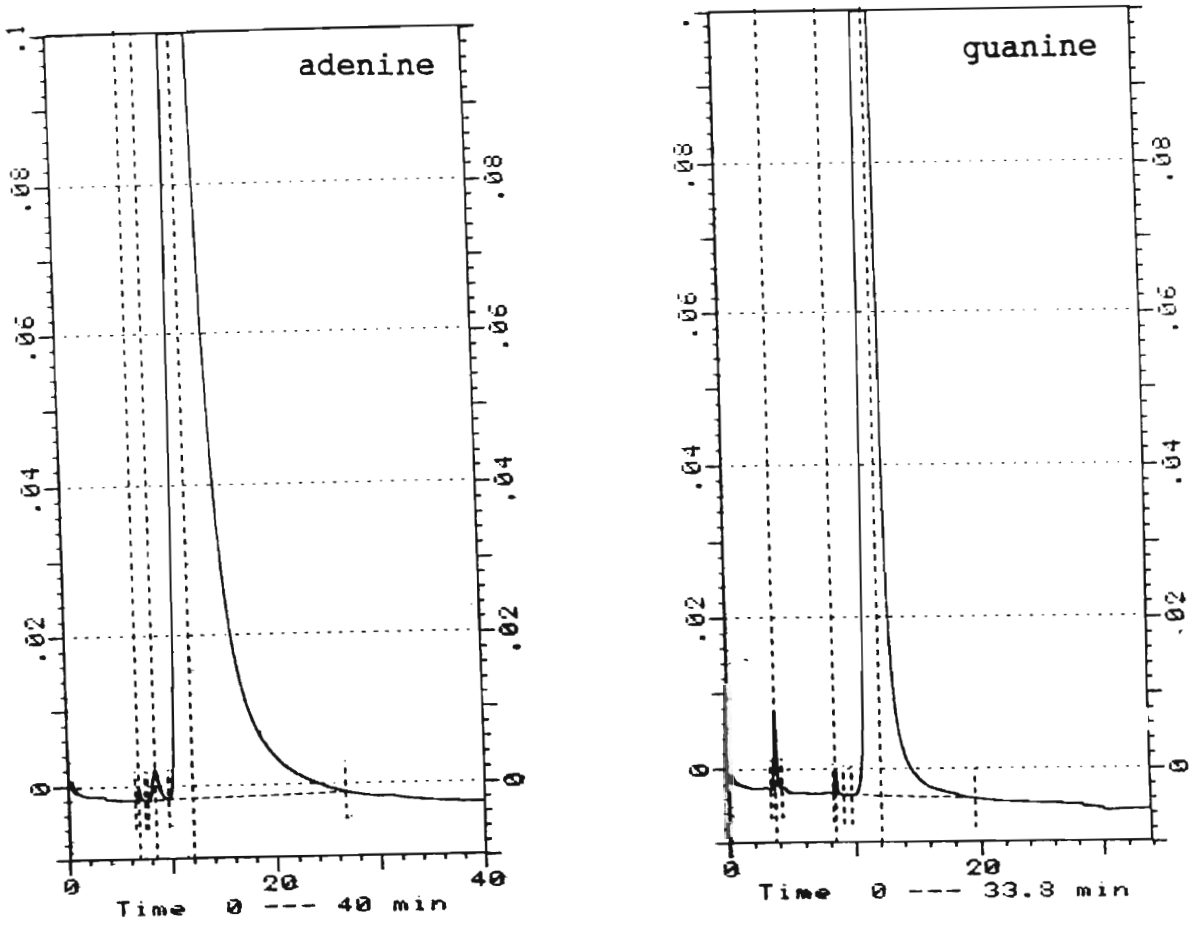


Figure 3.3. HPLC chromatogram showing retention times and UV spectra of spiked guanine and adenine solutions using a Milli-Q water (pH 3) mobile phase.

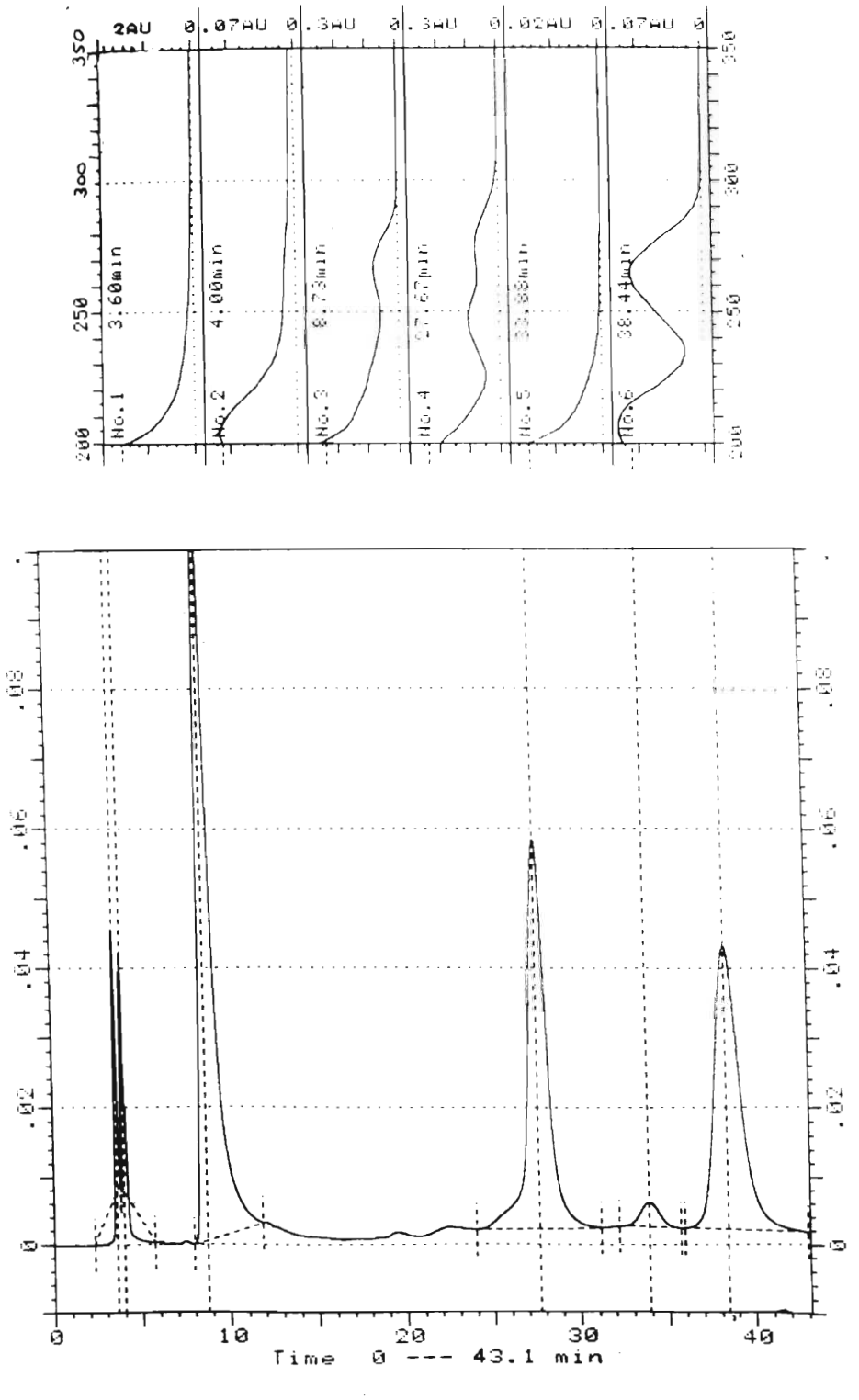


Figure 3.4. HPLC chromatogram showing separation of UV-irradiated DNA hydrolysate with Milli-Q water (pH 6) mobile phase.

appeared superior. This fact was substantiated by using 3% methanol in Milli-Q water (pH 3) which gave a resolution of the thymine monomer and dimer similar to that achieved for the Milli-Q water (pH 3) alone. Hence, Milli-Q water (pH 6) was used at a flow rate of 0.7 ml/min on an Ultracarb 5 ODS 30 column for the separation and quantitation of cis-syn thymine dimer from the thymine monomer in this work.

3.2.3 CONSTRUCTION OF A CIS-SYN THYMINE DIMER CALIBRATION GRAPH

A standard cis-syn thymine dimer calibration graph was required in order that dimer concentration (of prepared cis-syn thymine dimer) be related to dimer peak areas from HPLC analysis of irradiated DNA samples. The integrated dimer peak area of a particular component separated by HPLC is directly proportional to the concentration of that component present in the sample. Hence,

$$\text{peak area} = K \times \text{concentration}$$

where K is the constant of proportionality which is obtained from the gradient of the graph relating dimer peak area to dimer concentration. To construct such a graph, a series of cis-syn dimer solutions are required which fall into the expected range of dimer yields obtained by HPLC.

A stock solution of approximately 3×10^{-5} M cis-syn dimer was prepared by weighing out 0.00189 g of the prepared cis-syn thymine dimer and dissolving it in 250 ml of Milli-Q water, with heating to approximately 70°C. Successive dilutions of this stock solution were prepared and an aliquot of 30 μl of each solution was injected into the HPLC using the conditions outlined in the previous section. Three injections were performed for each solution and the peak areas integrated at 220 nm were averaged for each solution. The extinction coefficient of the dimer at 220 nm

was determined by Clemmett[114] to be 4 x 10^3 M^{-1} cm^{-1}. A calibration graph of dimer peak areas versus dimer concentration was constructed and linear regression of this calibration graph (shown in Figure 3.5.), yielded the following equation which was used to determine cis-syn dimer yields by HPLC anlysis in this work:

$$[TT] = \frac{PA + 19.9477}{1.22692 \times 10^8}$$

where [TT] refers to the cis-syn thymine dimer concentration as a molarity and PA refers to the integrated peak area at 220 nm.

Sections 2.8.1. to 3.2.3 have discussed irradiation techniques, DNA solution preparation and photoproduct analysis in irradiated DNA. These techniques were ones commonly used by members of this research group for analysis of irradiated systems.

Attention is now turned to the immunological aspects of this work. In Chapter 4, an introduction concerning the immunogenicity of DNA will be followed by a detailed discussion of the preparation of UV-DNA samples for immunization of rabbits. Thereafter, the immunization of rabbits will be discussed, followed by a discussion of the purification techniques of the serum (containing thymine dimer antibodies) produced by rabbits in this work.

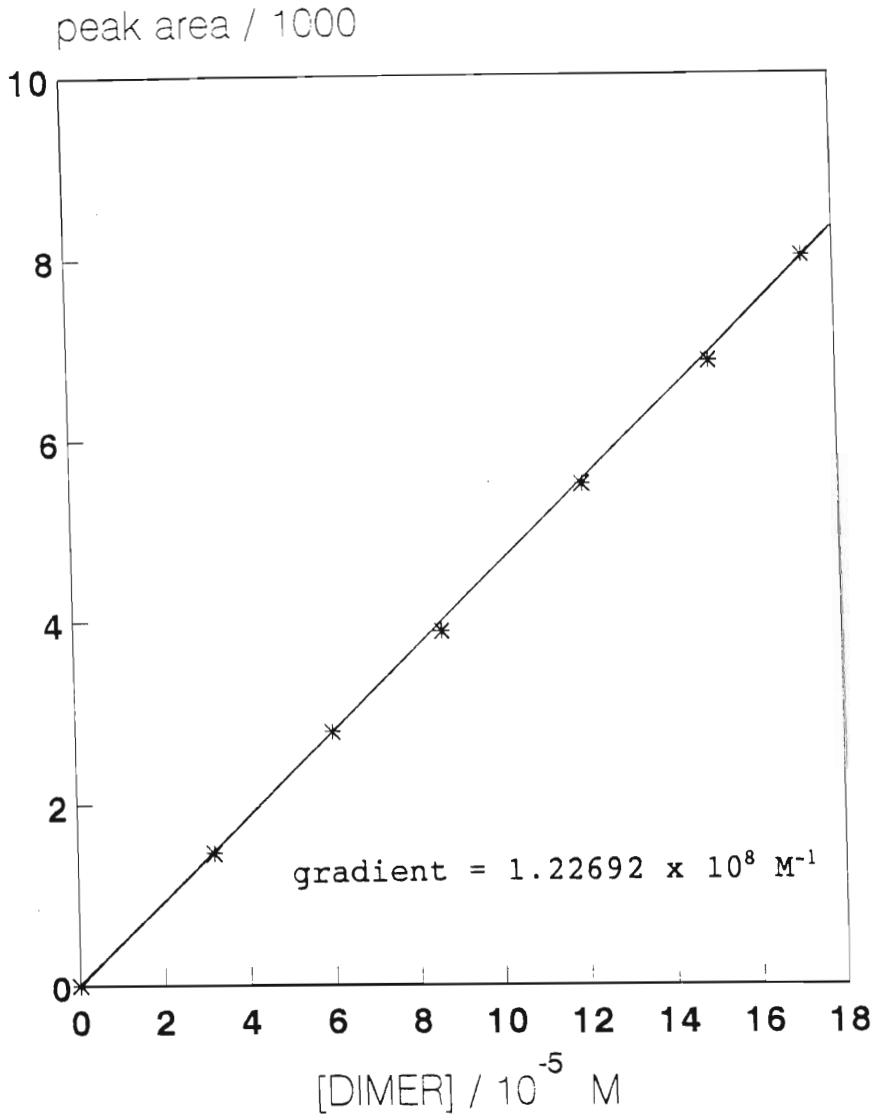


Figure 3.5. Cis-syn thymine dimer calibration graph used in calculating cis-syn thymine dimer concentrations.

CHAPTER 4 IMMUNIZATION TECHNIQUES

4.1 INTRODUCTION

Investigations into the production of anti-DNA antibodies in humans was initiated by Seligmann in 1957 who noted anti-DNA antibodies in the serum of patients with the disease Systemic Lupus Erythmatosus (SLE). This common auto-immune disease is characterized by inflammatory destructive changes in many organ systems, as well as the production of antinuclear antibodies. A rash of fixed erythema forming a butterfly pattern on the face is common with SLE patients, and this condition is worsened by exposure to sunlight. SLE may also be induced by drugs (e.g. Hydralazine) where the serum of these patients contain antibodies to histone protein[115].

For both idiopathic and drug related lupus, the basic question can be asked as to whether antibody production arises from an unusual presentation of the antigen or from an abnormality or alteration of the immune system itself[3]. Whichever option proves to be the correct one, the discovery of anti-DNA antibodies has opened up avenues of research which will eventually elicit information about therapeutic agents which will act by selectively modifying nuclear macromolecules, making them more immunogenic to the immune system.

Are nucleic acids immunogenic? In normal animals, both nucleic acids and histone proteins are poor immunogens when used as pure materials. If DNA and synthetic polynucleotides (double stranded RNA or DNA-RNA hybrids) are denatured, then the addition of protein carriers (see Section 4.2.1.4) renders the nuclear material immunogenic. If these same carriers are used in conjunction with native DNA, then no antibody production in response to the DNA is

noted. Chemically modified DNA, with an appropriate carrier molecule is strongly immunogenic, and it has been shown that modified structures are the major antigenic determinants in inducing antibody production. When histones are presented as histone-RNA complexes, they too, exhibit immunogenicity. It appears likely that antibody production in response to nuclear material, is strongly dependant on the physico-chemical state of the DNA and that antibodies may well be produced against modified conformations of the secondary, or higher, structure of the DNA rather than the DNA itself.

In helical double stranded DNA, all pyrimidine/purine bases are directed towards the central axis of the helix and are thus unavailable as antigenic determinants. Thus the deoxyribose-phosphate backbone is the major antigenic site when the molecule comes into contact with antibody producing cells. A single site could include a short segment of one or both backbones over a distance of about 3 base pairs[116]. Denaturation of DNA (achieved by rapid heating and cooling, or chemically induced - see Section 4.2.1.3) gives rise to a collapsed single stranded coil which may be presented to the antibody producing cells as loops or helices of deoxyribose-phosphate backbone. This is illustrated in Figure 4.1. In addition, many purine and pyrimidine bases are now exposed to solvent and available to act as determinants for those antibodies that react only with denatured DNA[117]. However, only the determinants for a few sera have been examined in detail. Included here is the thymine dimer which has been shown to be the antigenic determinant in UV-irradiated and denatured DNA[71] and which was injected into rabbits as UV-irradiated DNA in the work presented here. Wakizaka and Okuhara[81] have concluded that UV irradiation of DNA induces at least two different antigenic sites which are responsible for the production of two distinct antibodies. Moreover, their work indicated that conformational changes of DNA caused by irradiation,

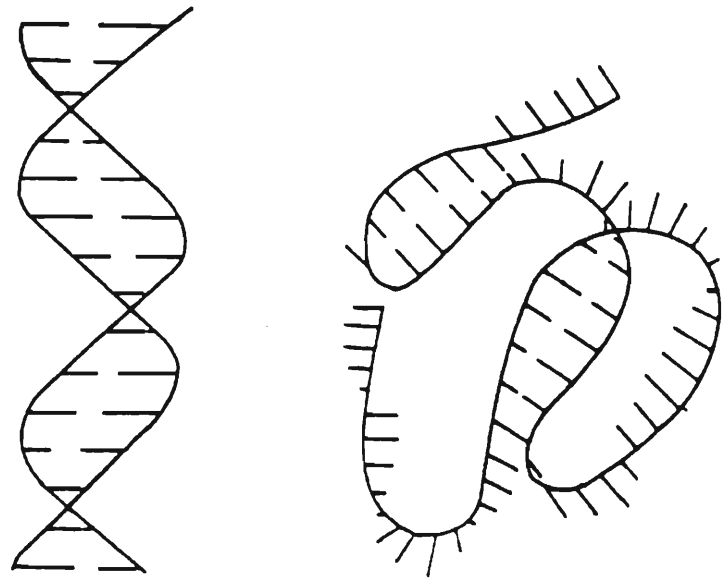


Figure 4.1. Diagram of DNA strands illustrating the purine and pyrimidine bases exposed to antibody molecules when DNA is denatured.

especially partial strand-separation of the double helical structure, greatly contributes to antigenicity. Considering the abovementioned aspects of the immunogenicity of DNA, the following section (4.2.1) describes the preparation of the irradiated DNA samples for immunization of rabbits in this work. First, the choice of DNA concentrations will be discussed. Thereafter, the use of acetophenone as a photosensitizer will be discussed briefly. Optimum denaturing conditions will also be evaluated, as well as the addition of methylated bovine serum albumin and Freunds Incomplete Adjuvant to the samples with which rabbits were immunized.

4.2 EXPERIMENTAL TECHNIQUES

4.2.1 PREPARATION OF UV-DNA SAMPLES FOR IMMUNIZATION OF RABBITS

4.2.1.1 Choice of DNA Concentrations

Although the DNA concentration used as the antigen for the immunization of laboratory animals is not crucial, it was important that the dimer concentration injected into the rabits be known, in order that antibody production be related to dimer yields. The DNA solutions of chosen concentrations would thus be UV-irradiated and the dimer yields analysed by HPLC. The hot acid hydrolysis technique as explained in Section 3.1.1 was optimized by Thomas[54] for the hydrolysis of 0.1 to 1.0 mg/ml DNA, and hence a range of DNA concentrations within these limits was chosen. It was also favourable not to create an "overload" situation of antigen in the rabbit tissue, although such a condition would have been difficult to detect during the immunizations themselves.

In order to determine irradiation times so that maximum thymine dimer yields could be produced, a series of time

runs involving 0.5 mg/ml and 1.0 mg/ml DNA with 2 x 10^{-2} M acetophenone were performed. Using the HBO 500W/2 short arc mercury lamp and the 10 mm Pyrex filter, 400 μl aliquots of DNA were irradiated for varying lengths of time. The samples were acid hydrolysed and analysed by HPLC using the Ultracarb 5 ODS 30 column and Milli-Q water (pH 6) at a 0.7 ml/min flow rate. Results are illustrated graphically in Figures 4.2. and 4.3. Maximum dimer yield of approximately 3.2 x 10^{-5} M was obtained for the 1.0 mg/ml DNA sample as evidenced by the plateau in Figure 4.2 after 10 hours irradiation. Maximum dimer production of 9.0 x 10^{-6} M was achieved for the 0.5 mg/ml DNA sample after 6 hours irradiation. Thus for injection of the 1.0 mg/ml DNA into the rabbits, the immunogen was irradiated for 10 hours, while for injection of 0.5 mg/ml UV-irradiated DNA a few months later, the DNA aliquots were irradiated for 6 hours.

Initially a 1.0 mg/ml DNA and 2 x 10^{-2} M acetophenone solution in PBS was prepared as a stock solution. A 400 μl aliquot was irradiated and denatured before being complexed to methylated bovine serum albumin and Freunds Incomplete Adjuvant. The addition of these other constituents thus effectively reduced the concentration of DNA injected to 0.72 mg/ml although dilution did not affect the number of thymine dimers presented to the rabbit immunocompetent cells. A solution of 0.5 mg/ml DNA was similarly prepared.

The immunization schedules that were employed in this work, as well as the problems that were dealt with, are discussed in Section 4.2.2.3.

4.2.1.2 Use of Acetophenone

As was explained in Section 2.8.2.4, acetophenone was added to all DNA preparations that were irradiated either for HPLC analysis, or prior to immunization of rabbits. Addition of this triplet photosensitizer ensured that the

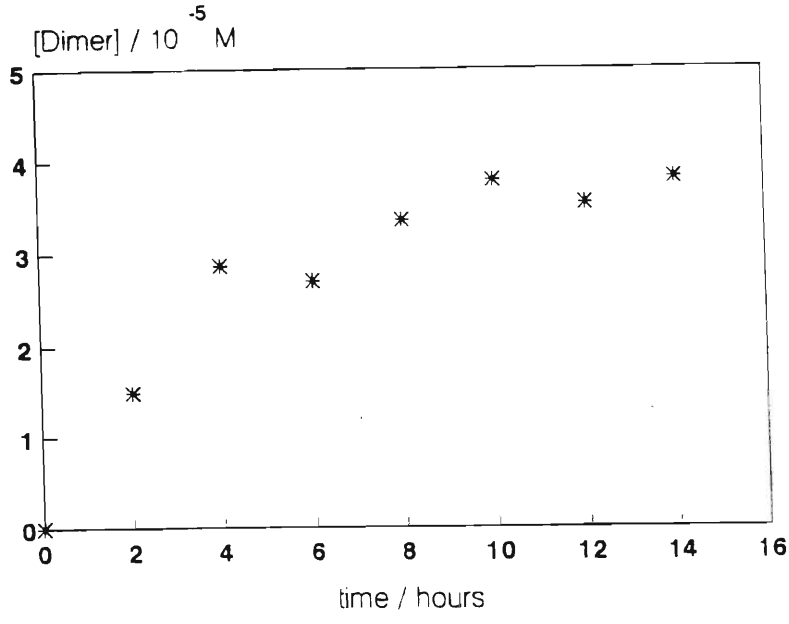


Figure 4.2. Cis-syn thymine dimer yields as a function of irradiation time with [DNA] = 1 mg/ml and [Acp] = 2 x 10^{-2} M.

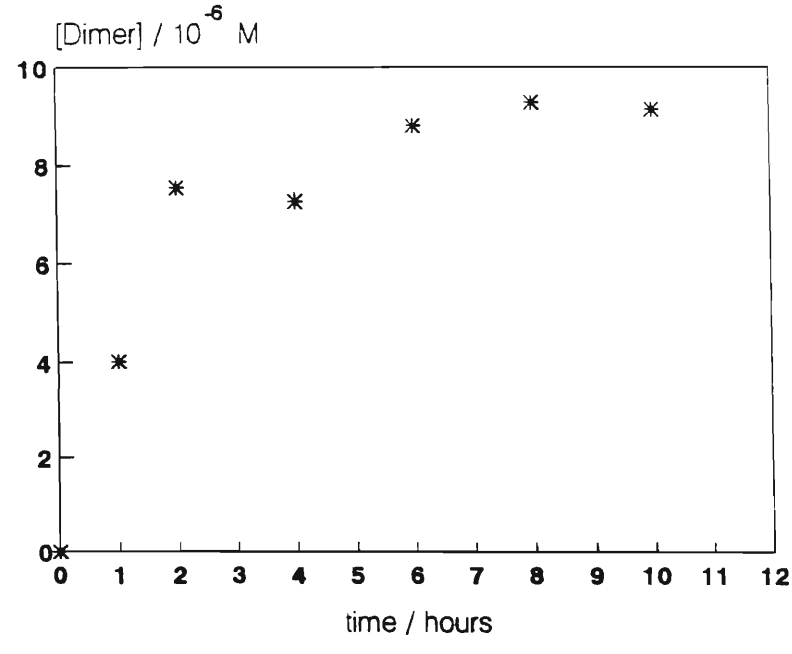


Figure 4.3. Cis-syn thymine dimer yield as a function of irradiation time with [DNA] = 0.5 mg/ml and [Acp] 2 x 10^{-2} M.

major UV photoproduct was the cis-syn thymine dimer, generated via indirect photosensitized irradiations of the DNA. Thus antibody production was directed against this photoproduct.

DNA-acetophenone-PBS solutions were prepared as outlined in Section 2.8.2.5 using a stock solution containing 0.15 M PBS buffer and 2 x 10^{-2} M acetophenone. A 400 μl aliquot of the prepared DNA solution was transferred to a quartz irradiation cuvette and irradiated for the chosen irradiation period using the HBO 500W/2 mercury arc lamp. At the end of the irradiation period, the sample was transferred to a Durham tube and denatured as oulined in the following section.

4.2.1.3 Determination of Optimum Denaturation Conditions

Denaturation may be described as the reversible or irreversible change in the properties of proteins or nucleic acids. This process is most commonly effected by the addition of heat or chemicals to a protein or DNA preparation, which brings about changes to the secondary, and maybe tertiary and quaternary structure, of the compound.

To understand how denaturation affects the DNA macromolecule, it is imperative that the stabilizing forces within the molecule be explained. The stability of the Watson-Crick double helix structure depends primarily on the associative hydrogen bonds between purine and pyrimidine bases, as well as hydrophobic interactions between the bases themselves due to stacking. In addition, a further stabilizing influence is provided by cavitation energy; that is energy supplied by the hydrogen bonding of the deoxyribose-phosphate backbone to a shell of water molecules. From an ionic point of view, counter ions such as Na^{+} and Ca^{2+} neutralize the negative charges on the

backbone of the polynucleotide.

If sufficient energy is provided to the DNA to overcome its melting temperature, or if a chemical preparation is added (1 M NaOH, 0.01% to 1.0% formaldehyde[118]) to the DNA, then loss of these stabilizing factors brings about loss of the secondary structure of the DNA and denaturation is brought about.

Spectroscopic analysis has been used to monitor the denaturation process[118]. DNA absorbs strongly at 260 nm due to absorption of this radiation by the conjugated double bond systems of the constituent purines and pyrimidines. If destacking of these bases occurs (i.e. if the DNA is denatured), an increase in UV absorption is noted at 260 nm proportional to the extent of denaturation. This phenomenon is callled the hyperchromic effect[119].

In this work, denaturation was effected by heating the DNA. Alternative denaturation routines such as the addition of formaldehyde, glycols, alcohols or sulphoxides[120] to the DNA preparation, could have complicated the nature of the antibody binding sites of the antibodies, hence reducing specificity.

Doty et al.[121] first noted that slowly heated DNA undergoes a co-operative melting out of the helical structure yielding disorganized and coiled polynucleotide chains. At a 0.02 M concentration of Na^+ ions, the midpoint of this transition (melting temperature) lies in the 80 to 100°C range depending on the guanine-cytosine content of the DNA (guanine-cytosine bonds are stronger than thymine-adenine bonds). These researchers also noted that two different molecular states of the DNA are obtained depending on the rate of cooling of the DNA, the DNA concentration and the ionic strength of the solvent. If the DNA is heated and then fast cooled, the DNA consists of

single strands having approximately half the molecular weight of the original preparation[118]. If the DNA is heated and cooled slowly, then it consists of recombined strands united by complementary base pairing over most of its length. These two states of the DNA are illustrated in Figure 4.4., together with other hypothetical conformations which may also exist. The heated and slowly cooled DNA is called renatured DNA, while the former denatured state is essentially inactive. These forms can clearly be distinguished by differences in absorbance temperature curves (see Figure 4.5.), density, appearance in electron micrographs and hydrodynamic properties.

Several researchers have noted that the dimerization process brings about concomitant changes to the higher structure of DNA. Ciarrocchi and Sutherland[30] postulate that photodimerization of thymines in DNA induces an unwinding of the helical structure of DNA. Using X-ray diffraction studies, the unwinding angle was calculated to be 8°. The use of sensitizers during irradiation has also been implicated in altering secondary DNA structure. Charlier and Helene[122] proposed that the hydroxy radical formed during photolysis in the presence of the photosensitizer benzophenone is responsible for observed chain breakage. The use of acetophenone and acetone has been shown to induce fewer strand breaks than benzophenone[123]. Thus localized denaturation of DNA is induced during irradiation, yet these features are insufficient in maximally exposing the thymine dimers to immunocompetent cells.

It is advantageous for the DNA presented to the rabbit immune system to be in the single strand form. This facilitates exposure of the thymine dimers (located towards the central axis of the DNA) to the antibody producing cells. Seeing that calf thymus is double stranded, it was imperative to denature the DNA. From a practical

I

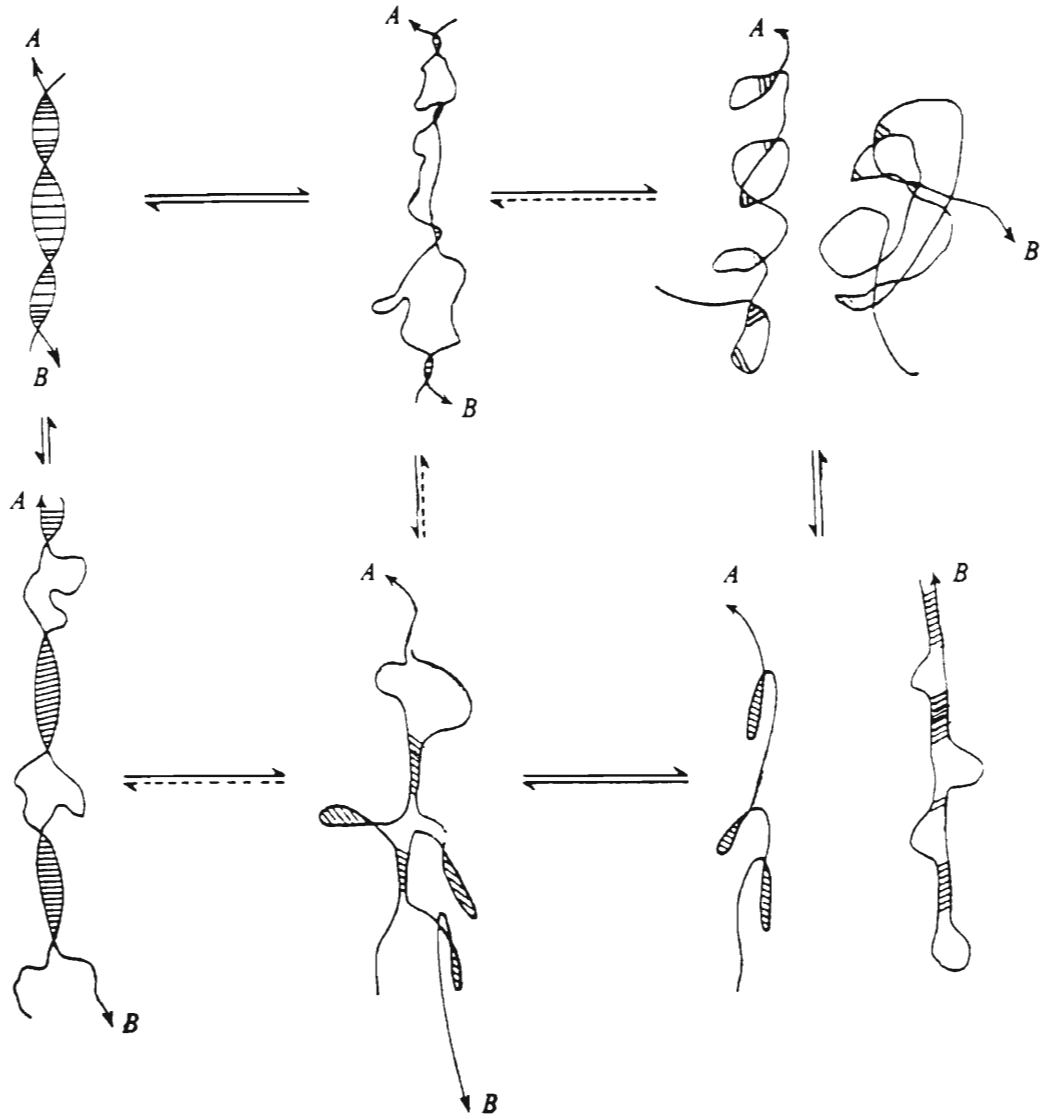
Double helix (room temp, aqueous, neutral *p*H)

IV

Disordered (random coil) regions almost exclusively, but strands still entangled in register

V

Separated random coils



II

Disordered and ordered regions, strands in register, ordered regions may no longer contain H bonds

III

Disordered and ordered regions, strands not in register, base pairs other than AT and GC possible, as is stacking without H bond formation

VI

Separated coils with considerable intramolecular helicity (two extreme cases shown)

Figure 4.4. Hypothetical conformations of denatured DNA strands[120].

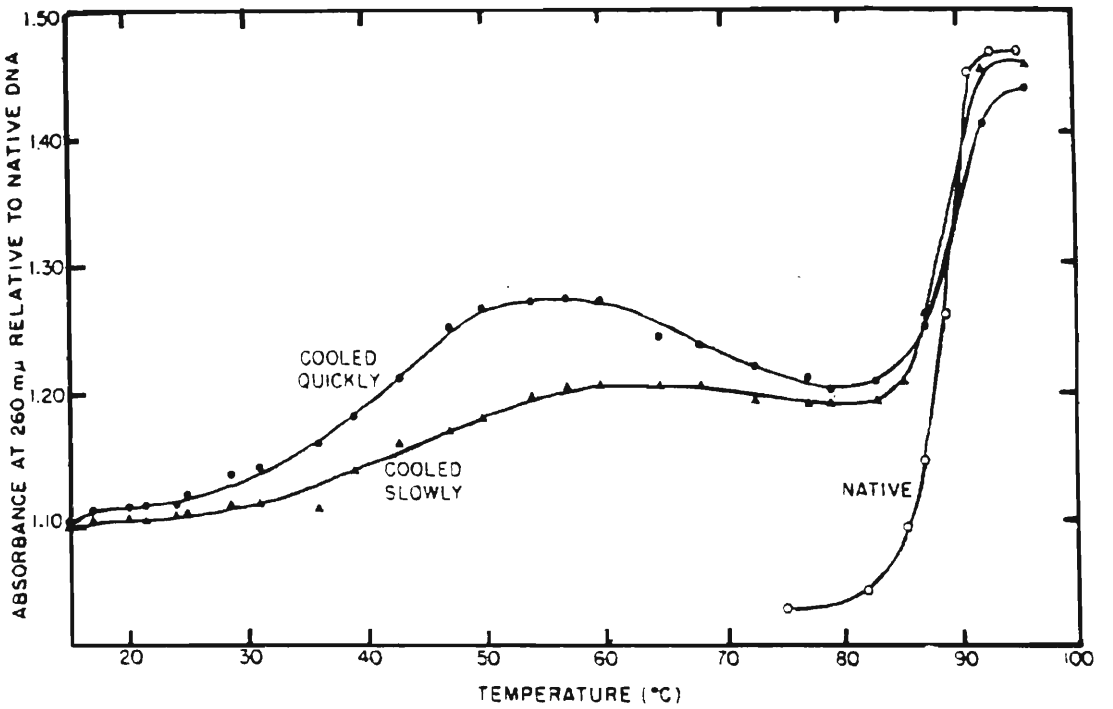


Figure 4.5. Thermal transitions of native, slowly cooled and quickly cooled DNA[121].

consideration, complexation of methylated bovine serum albumin (MBSA, Section 4.2.1.4) with native DNA results in a compact fibrous clot that is impossible to inject. If the DNA is denatured, then the precipitate formed between the carrier protein and DNA is granular and dispersed and is injected without difficulty.

Leipold et al.[82] performed denaturation of a 0.5 mg/ml DNA sample by boiling the double stranded nucleic acid for 10 minutes and then rapidly chilling the sample on ice. This technique was also performed by Wani et al.[124], Plescia, Braun and Palczuk[66], as well as Doty et al.[121]. It was important in this work that the denaturation technique yield a fully denatured (as opposed to a partially denatured) preparation. For highly purified DNA, increases in A_{260} (absorbance at 260 nm) of as much as 40% are indicative of a fully denatured sample[119]. Hence a series of denaturing conditions were tested, in order that fully denatured samples be prepared. A 0.1 mg/ml DNA solution in PBS was prepared and 400 μl aliquots were heated on a polyethylene glycol (PEG) bath at 100°C for varying lengths of time (10, 20 and 30 minutes). After heating, the samples were rapidly transferred to crushed ice for 10 minutes.

A 10 minute heating period gave a 41% increase in absorbance at 260 nm, while the 30 minute period conferred a 72% increase in absorbance at 260 nm. Erratic results for the increase in absorbance after 20 minutes were obtained and hence this heating period was excluded. The increases in absorbance after 10 and 30 minutes are illustrated in Figure 4.6. Seeing that full denaturation was achieved by heating the DNA sample for 10 minutes at 100°C, this heating period was chosen to denature the UV-DNA immunogen in accordance with the denaturation as performed by Wani et al.[124], Plescia et al.[66] and Leipold et al.[82]. During denaturation in the PEG bath, an aluminium foil seal which contained numerous pin pricked holes covered the Durham

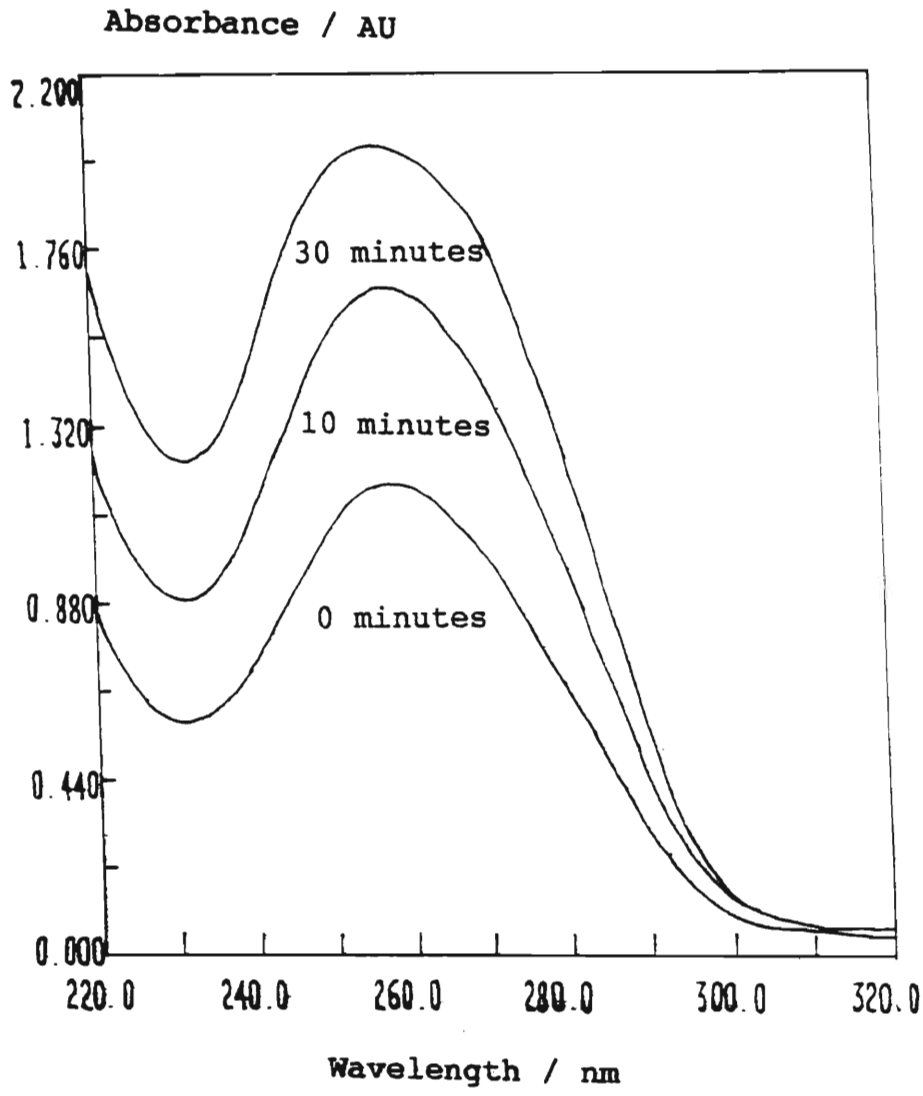


Figure 4.6. Absorption spectra of 0.1 mg/ml DNA illustrating an increase in absorption at 260 nm following selected heating denaturation periods.

tube containing the DNA sample. This allowed evaporation of the acetophenone during denaturation. Heating was followed by rapid transferral of the Durham tube (using tweezers) to a beaker of crushed ice for 10 minutes. When the tube and contents had come to room temperature, the sample was transferred using a micropipette into a sterile 12 ml Polytop glass vial (vial number 3). Thus the DNA strands were separated during heating and the rapid cooling maintained this separation and hence ensured maximum exposure of the thymine dimer to the rabbit immunocompetent cells.

A total volume of 2.2 ml was injected into laboratory rabbits by Leipold et al.[82], with the DNA-MBSA being in a 1:1 ratio with the Adjuvant. Following this protocol, 660 μl of sterile Milli-Q water was added to the contents of the Polytop vial to yield 1.10 ml of diluted DNA complex after addition of MBSA. The water was added in 3 aliquots, which were first added to the Durham tube and the washings then added to the contents of the sterile glass vial. The vial was sealed and stored in the refrigerator if this solution was not to be used immediately.

The following section descibes how the irradiated and denatured DNA samples were conjugated to the protein carrier molecule methylated bovine serum albumin in order to facilitate antigen transfer across cell membranes after immunization.

4.2.1.4 Complexation to Methylated Bovine Serum Albumin

Carrier molecules enable the antigen to be taken up by immunologically competent cells and thus participate in the induction of antibodies specific for the antigen. This requirement of absorption of the carrier-antibody complex can be performed by any heterologous protein, however consideration must be given to the fact that conjugation to

a non-immunogenic polymer may render the polymer immunogenic and thus induce antibodies against it. A second consideration in the choice of a carrier is the feasibility of forming a conjugate between the antibody and the carrier.

In the case of proteins, conjugation is easily achieved through reaction of the antigen with free $-NH_2$ and -OH groups of amino acids to produce covalent linkages to the nucleic acid. However, several other linkages have also been employed for this purpose. Halloran and Parker[125] described the use of water soluble carbodimide reagents to conjugate nucleotides (e.g. thymidylic acid) to proteins, potentially through a phosphoramide linkage. In this and other cases, antibodies were generated against both the antigen and the heterologous carrier proteins, but there are problems associated with achieving covalent complexes between nucleic acids and the carrier proteins.

This problem of achieving covalency led Plescia et al.[66] in 1964 to postulate the formation of electrostatic complexes between DNA and carrier molecules: DNA is an acidic polymer which interacts readily with basic proteins under physiological conditions to form stable complexes. Bovine serum albumin was used exclusively as a carrier protein and it seemed plausible that methylated bovine serum albumin (MBSA) could be used as a carrier for DNA. Results showed conclusively that antibodies would be produced against antigens (e.g. DNA) linked to the MBSA through non-covalent, but nevertheless stable bonds. This general methodology of producing complexes through electrostatic interactions has subsequently proved to be effective not only for nucleic acids, but also for the production of antibodies against certain polypeptides and polysaccharides that are ordinarily not immunogenic in chemically pure form[126]. Seaman et al.[68] showed that MBSA is also an effective carrier for synthetic polynucleotides

including Poly (A), Poly (I) and Poly (C). Levine et al.[71] have shown MBSA to be an effective carrier for UV-irradiated DNA and Seaman et al.[127] have shown it to effective for photo-oxidized DNA.

If this technique of forming electrostatic interactions is employed, the nature of the interaction as well as the groups involved appears to be critical. DNA-MBSA elicits a specific antibody against DNA[66], while conjugates of DNA-BSA (formed by hydrogen bonds) fail to do so[68].

Apart from facilitating uptake of the antigen by antigen presenting cells, complexes of polynucleotides and MBSA are apparently resistant to nuclease attack[66]. It is postulated that this resistance to enzyme attack may be important for the efficacy of MBSA as a carrier for polynucleotides. Sela[128] has proposed that this carrier moeity, while not contributing to the specificity of antibody production, plays an important role in defining the biosynthesis and structure of the antibodies formed.

Plescia et al.[66] mixed solutions of native DNA and MBSA, to find that a compact fibrous precipitate resulted. If heat denatured single stranded DNA and MBSA were mixed, fine particles formed which did not dissociate under physiological conditions and were highly resistant to the action of DNAse. Seeing that irradiated and denatured DNA was being used as the antigen in this work, it was apparent that MBSA would act as an efficent carrier of the DNA antigen.

The stability of this DNA-MBSA complex depends primarily on the concentration (and hence pH) and nature of the buffer used as a diluent. Dissociation of the DNA-MBSA complex occurs at high and low pH and at salt concentrations greater than 0.15 M. Thus the PBS buffer (pH 7.35) in which the DNA was dissolved, provided a suitable diluent for the

DNA-MBSA complex. Because MBSA dissolves more easily in water than in 0.15 M NaCl, the MBSA was added to irradiated and denatured DNA samples as an aqueous solution.

The MBSA was supplied by Sigma and was prepared according to the method of Sueoka and Cheng[129]. This involved methylating BSA with absolute ethanol in an HCl environment. The resulting feathery compound was stored in a dessicator in a refrigerator. A 1% solution was prepared by Plescia et al.[66] and this concentration has been used in all subsequent work on the immunization of laboratory animals with DNA components. In this work, the solution was prepared using sterile glassware and sterile Milli-Q water. Here, 0.2507 g of MBSA was weighed out and transferred to a beaker containing approximately 20 ml sterile water. Despite the fact that the MBSA was light and feathery, it dissolved easily with swirling. The contents of the beaker were then transferred to a sterile 25 ml volumetric flask and made up to the mark using sterile Milli-Q water. The solution was stored in a refrigerator. Seven months later, more 1% MBSA solution was prepared because of visual bacterial comtamination of the original MBSA solution.

When the MBSA solution was required, it was brought to room temperature. Plescia et al.[66] suggested a 1:1 weight ratio of DNA:MBSA to be used. For a 400 μl aliquot of 1 mg/ml irradiated and denatured DNA, the corresponding mass of MBSA was present in a 40 μl aliquot of 1% MBSA. This volume of MBSA was transferred to the DNA solution contained in the sterile 12 ml Polytop glass vial using a 20 to 200 μl micropipette. The solution became cloudy as the ionic conjugate was formed.

Although the DNA-MBSA conjugate could have been injected into the rabbits at this stage, enhanced antibody production has been achieved if the conjugate is supplied to the rabbit in the presence of an adjuvant (usually

Freunds Complete or Incomplete Adjuvant). The following section describes the usage and advantages of adjuvants in immunization schedules and discusses the use of the adjuvant employed in this research.

4.2.1.5 Use of Freunds Incomplete Adjuvant

When dealing with limited amounts of protein or nucleic acid as antigen or when the immunogen is a weak one (e.g. nucleic acids), it is advantageous to employ techniques that enhance the immune response. By facilitating transport of antigen to immunocompetent cells (see previous section), the use of MBSA has the function of enhancing antibody production. However, greater enhancement of antibody production is commonly effected by the proper use of adjuvants.

From an immunological perspective, the use of adjuvants potentiates the humoral antibody response to the injected antigen. Among the most common adjuvants are inorganic adsorbents such as mineral oils, aluminium hydroxide gels and bacterial cell wall components. Because of the diversity of materials having adjuvant properties, the specific mode of action of the adjuvant is not well understood. Three major effects are, however, involved albeit to different degrees for each adjuvant type. The most important effect concerns a slowing of the release of the immunogen from the site of injection. This is thought to occur by either adsorption onto solid particles (as in the case of aluminium hydroxide) or by incorporation into an oily emulsion. Thus by adsorption onto the adjuvant, the emulsion forms a "depot" of antigen in the host tissue from which small quantities of antigen are continually released, sometimes for a year or more (Figure 4.7.). This allows more antibody forming cells to be exposed to the limited amount of antigen and because the antigen is contained within the emulsion, local destruction and elimination of

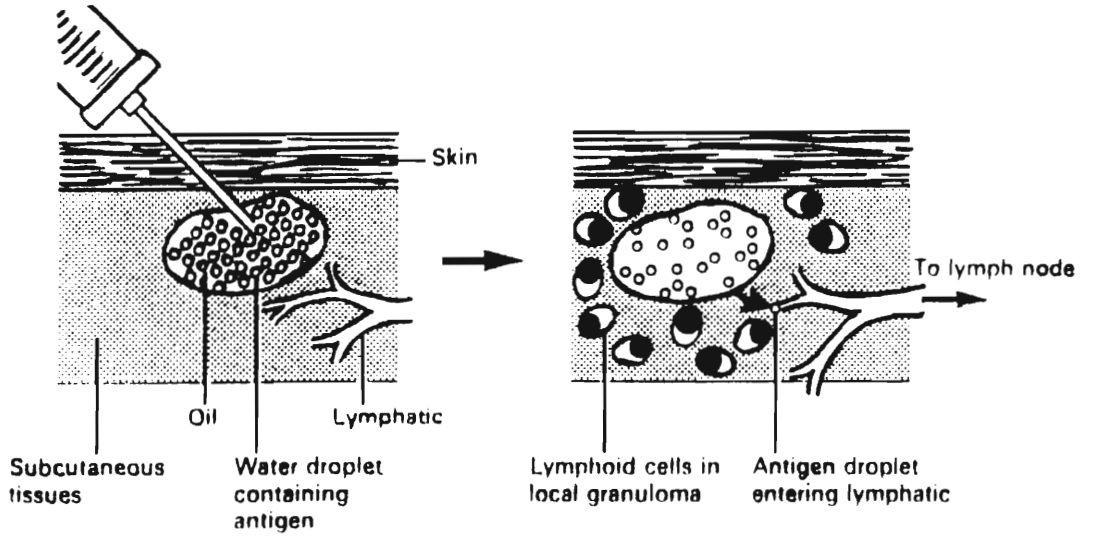


Figure 4.7. Diagram illustrating the mode of action of Freunds Adjuvant.

the antigen by proteolytic enzymes is retarded.

A second function of adjuvants is thought to involve a stimulatory effect on reticuloendothelial cells. The addition of the adjuvant attracts a local infiltration of the injection area by mononuclear cells and thus stimulates phagocytosis (endocapsulatory destruction) of the antigen by the macrophages. It has also been shown that adjuvants induce an increased circulation of lymphocytes (involved in antibody production) through lymphoid tissues in the drainage area, macrophages being important for the initiation of the changes of lymphocyte traffic.

In 1942, Freund and co-workers[130] developed a series of adjuvants containing mycobacteria, mineral oil and emulsifier, which today remain the most potent adjuvants known. The most widely used mixture contains 9 parts light mineral oil (e.g. Bayol) to 1 part detergent (e.g. Arlacel A). The detergent usually contains a high level of both lipophilic and hydrophobic groups facilitating the formation of a stable emulsion. These simple oil-detergents are known as Freunds Incomplete Adjuvants. The addition of heat killed mycobacteria (*Mycobacterium butyricum* or *M. tuberculosis*) at a 0.5 mg/ml concentration to the oily mixture is referred to as Freunds Complete Adjuvant and is far more effective in eliciting an immune response, presumably by increasing local inflammatory responses due to the mycobacteria. Despite these much touted advantages of enhancing antibody production over a period of time, it is also possible that the emulsion might destroy some of the antigenic determinants of antigens[131].

The Freunds Adjuvant employed in this work was supplied by Sigma as "Freunds Incomplete Adjuvant". An incomplete adjuvant was chosen because this compound would enhance, but not artificially elevate, antibody titres in the rabbit serum.

The Incomplete Adjuvant, containing 15% mannide monoleate in 85% paraffin oil, was supplied as a series of 10 ml ampoules. These were stored in a refrigerator. When required, the adjuvant was brought to room temperature and a sterile 2.50 ml hypodermic needle was used to withdraw a 1.10 ml aliquot of the oily suspension. This was then transferred to the DNA-MBSA solution in individual Polytop glass vials. The addition of the 1.10 ml aliquot of adjuvant ensured that a 1:1 volume ratio of DNA-MBSA to Adjuvant was achieved. The system immediately formed two immiscible layers which were difficult to inject into the rabbit ear vein. However, this problem was overcome by the staff of the Biomedical Resources Centre (University of Durban-Westville) who aspirated the oily emulsion prior to immunization using two syringes.

As will be explained in Section 4.2.2.3, 8 individual UV-irradiated DNA immunogens (containing both MBSA and Freunds Incomplete Adjuvant) were administered over a 4 week period to each of 2 rabbits. All 8 samples were transported to the Biomedical Resources Centre at the University of Durban Westville where they were stored in a refrigerator before being administered at the appropriate time.

This section (4.2.1) has elucidated the means by which a UV-DNA antigen was prepared for immunization into laboratory animals. This involved denaturation of the DNA (allowing maximum exposure of the thymine dimer antigenic determinants), complexing to MBSA (facilitating transfer of the antigen to immunocompetent cells) and adding Freunds Incomplete Adjuvant (enhancing antibody production of the poor DNA antigen).

The following section (4.2.2) describes the choice of laboratory animal used for immunization of the UV-irradiated DNA antigen, the immunization schedule that was followed in this work, as well as the means of bleeding the

rabbits in order to obtain the serum containing the thymine dimer antibodies.

4.2.2 IMMUNIZATION OF RABBITS

This section describes the immunization and bleeding of the laboratory animals used in this work. First, the choice of animal to which the UV-irradiated DNA antigen was administered will be discussed. Then the housing of the laboratory animals will be discussed, followed by the injection routes and immunization schedules that were chosen. Finally, the bleeding of the animals (to obtain the antibodies generated against the UV-irradiated DNA) will be discussed.

4.2.2.1 Choice of Animal for Immunization

There are few instances in the literature where one species of laboratory animal gives consistently better responses than another to a particular immunogen[132]. In most cases where a choice of laboratory animal must be made, the decision usually depends on the animal availability and the volume of serum required. Usually the larger the animal the higher the yield of serum. It is also sensible to immunize a species of animal that has never been exposed to the antigen in question.

With these considerations in mind, rabbits are usually the first choice for immunizations unless very large volumes of sera are required. From a practical point of view, rabbits are cheap to house and feed, easy to care for and bleed, and are particularly robust when immunized repeatedly. An alternative species is the chicken, which is also easy to handle, but the antibodies that it produces behave differently from those of mammalian species and are thus best avoided.

To date, complexes of DNA and MBSA have been tested in two species, the rabbit and the mouse. Antibodies were produced by the rabbit to both DNA and MBSA, whereas the mouse proved unable to produce antibodies against either[134]. This lack of response was noted in several strains of mice, so appears to be general for this species. It was thus sensible to use rabbits as the host animal for immunization.

New Zealand white male rabbits were supplied by the Biomedical Resources Centre at the University of Durban-Westville and all immunizations and bleeds were performed by their staff. New Zealand white rabbits were used by Wani et al.[124], Natali and Tan[75] and Plescia et al.[66] while Levine et al.[71] and Leipold et al.[82] made mention of using between one and nine rabbits for the production of anti-DNA antibodies in rabbits. In this research, three rabbits were used for immunization purposes over the period of a year.

4.2.2.2 Housing of Rabbits

Each of the three rabbits used was housed in a fibreglass rabbit cage suspended over a stainless steel tray containing dust free woodshavings as bedding material. The animals were housed in purpose built animal rooms at the Biomedical Resources Centre. The room temperature was maintained at 21°C while the relative humidity was controlled at 50%. Air ventilation was maintained at 15 to 20 air changes per hour by means of an airconditioner. A 12 hour light cycle followed by a 12 hour dark cycle was maintained using electrical light switches. The animals' daily diet of compressed food pellets was supplemented with hay twice weekly and they had access to fresh water whenever it was required.

4.2.2.3 Injection Routes and Immunization Schedules

Injections (subcutaneous, intravenous and intramuscular) were administered to the rabbits by the staff of the Biomedical Resources Centre. They were performed using 25 gram one inch needles attached to 25 ml disposable plastic syringes. Subcutaneous delivery of the antigen was performed under a loose fold of skin behind the neck of the rabbit. Intravenous injections were performed using a marginal ear vein, while intramuscular injections were performed using the hind leg musculature of the rabbit.

Initially two rabbits received the UV-irradiated DNA antigen, one via a subcutaneous and the other by an intravenous injection route. The 1 mg/ml antigen was administered to both rabbits once a week over a four week period using the abovementioned injection routes. However, it soon became obvious that intravenous injection of the antigen (containing UV-irradiated DNA, MBSA and Freunds Incomplete Adjuvant) was not successful, primarily because the viscous antigen was difficult to inject into the ear vein of the rabbit; the vein kept closing up and had to be rubbed repeatedly during injection. Hurn and Chantler[131] also note that administration of Freunds Adjuvant via an intravenous route can induce the condition of fatal fat embolism. Cardiac puncture was thus performed on this rabbit and the large volume of serum that was obtained was used in the serum purification protocol using affinity chromatography (see Section 4.2.3.4 to 4.2.3.10) as well as the developmental stages of the immunoblotting protocol (Section 5.2.1).

Four months after the final subcutaneous injection of the 1 mg/ml antigen, the same rabbit received a further series of subcutaneous injections (1 mg/ml). In order to compare antibody production via injection routes, a third New Zealand white male rabbit received a series of

intramuscular injections of the 1 mg/ml UV-irradiated DNA (complexed to MBSA and Freunds Incomplete Adjuvant). As previously, 4 injections were administered to each animal over a four week period.

In order to keep a high antibody titre in the rabbit serum, a further series of subcutaneous and intramuscular injections were performed on the same rabbits using a 0.5 mg/ml UV-DNA antigen. The first injection was performed approximately 50 days after conclusion of the previous injection schedule.

4.2.2.4 Bleeding of Rabbits

In order to obtain the antibodies generated against thymine dimer, blood collections were performed using the marginal ear veins of the rabbits. Bleeds were performed prior to each injection (thus 4 bleeds gave 4 serum samples during the injection schedule) as well as 10 and 20 days after completion of the fourth injection. The very first of the bleeds was a control bleed, while the fifth and sixth bleeds monitored antibody production once antigen injections had ceased.

When bleeds were performed, the animal was restrained in a purpose built rabbit restraining box and did not require the use of anaesthetics. The blood sample (usually 2 to 5 ml) was stored in a 5 ml test tube in a refrigerator overnight in order to clot the blood. The following morning the clotted blood sample was centrifuged at 3500 rpm for 15 minutes, after which the serum (containing the thymine dimer antibodies) was pipetted into 1.50 ml Eppendorf vials. The vials were labelled and frozen in the freezer compartment of a refrigerator until immunoblotting analysis or serum purification was performed.

The following section (4.2.3) describes the purification

regimes that were employed in this work. First, the precipitation of protein from crude serum using saturated ammonium sulphate solutions will be discussed. Then, the theoretical aspects of affinity chromatography, employing a commercial Memsep cartridge, will be explained and following that the developmental work for antibody purification using this cartridge will be discussed.

4.2.3 PURIFICATION OF CRUDE SERUM

This section describes techniques for the purification of the serum containing antibodies to thymine dimer. First, ammonium sulphate precipitation of immunoglobulins will be discussed and evaluated as a purification technique. Then, the technique of affinity chromatography will be discussed in terms of a protocol chosen to provide reproducible purification of the antibody containing serum.

4.2.3.1 Characterization of the Crude Serum by UV Spectroscopy

A Varian DMS 300 UV/Vis double beam spectrophotometer together with Hellma 50 μl ultracuvettes (shown in Figure 4.8.) was used to obtain UV spectra of the crude rabbit sera. The serum components of vertebrates are not restricted to antibodies, serum proteins (transferrin, albumin and cytochrome) are also present and serve important functions such as iron transport within vertebrate tissues. All of the serum components (antibodies and serum proteins) are obviously proteinaceous or glycoproteinaceous in nature. Hence, a UV spectrum of a serum sample will not distinguish between the antibodies or serum components. However, UV spectra were run and compared against the spectra obtained from commercial rabbit IgG (the most prolific antibody comprising 80% of the total antibody production). This served to confirm whether antibodies were generated during and after the immunization

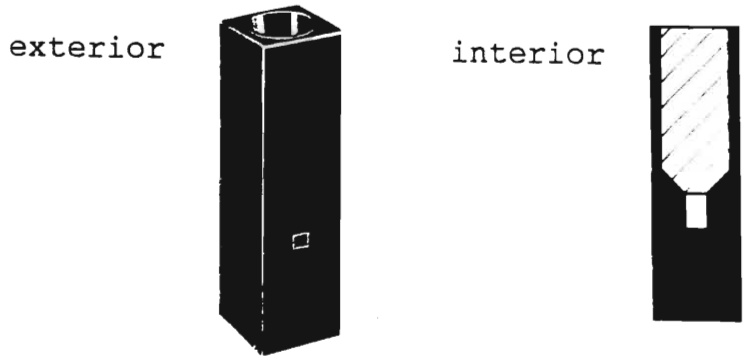


Figure 4.8. Suprasil quartz 50 µl Hellma ultracuvette.

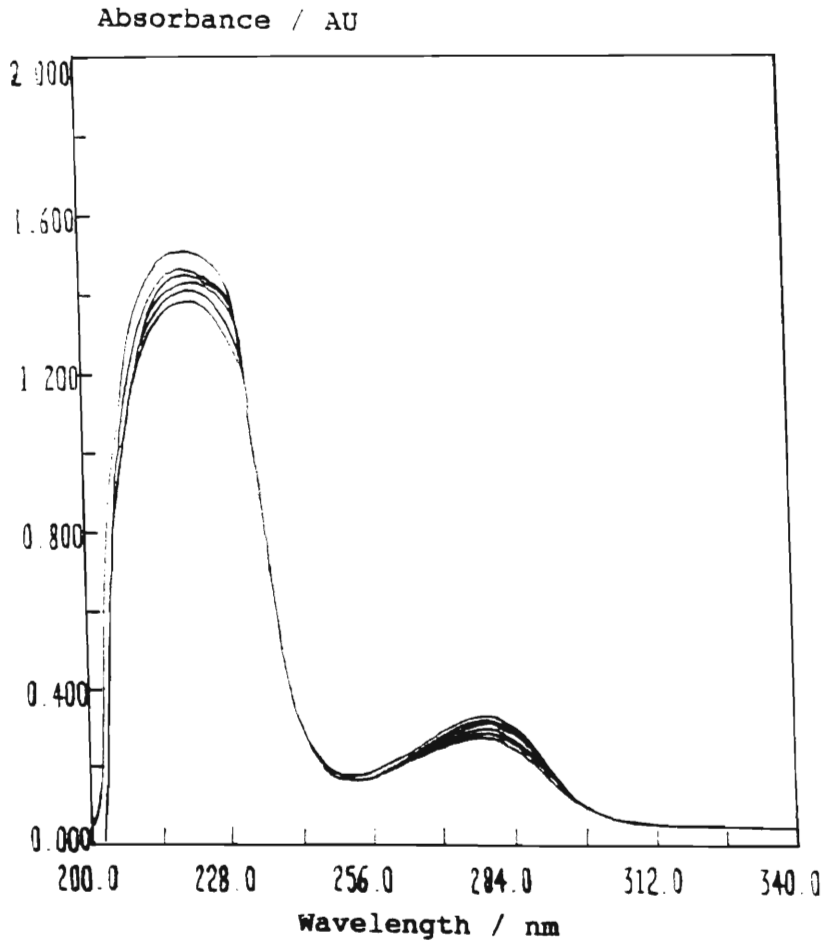


Figure 4.9. Absorption spectra of serum samples (1:200 dilution) taken from 6 successive bleeds of the rabbit.

schedules and the results are discussed below.

Figure 4.9. shows six UV spectra (1:200 dilutions of each serum sample), one from each of 6 successive bleeds during and after the immunization of the rabbit with the UV-irradiated DNA antigen (see Section 4.2.2). All spectra have the characteristic UV absorption maxima at 278.3 nm to 279.1 nm and 216.9 nm to 219.7 nm. The absorption at 278.3 nm to 279.1 nm is associated with the tyrosine and tryptophan amino acid content of the antibody and serum proteins. More specifically, it is the conjugated double bond systems of tryptophan (Figure 4.10.) and tyrosine (Figure 4.11.) which provide the absorption. Tryptophan has λ_{max} at 278 nm[133] which is not affected by changes in pH, while tyrosine has λ_{max} at 274.5 nm[133] which is affected by ionization of the -OH group in alkaline solution. Thus the PBS diluent used here (pH 7.35) has marginally affected the λ_{max} due to the tyrosine absorption. The absorption in the far UV at 216.9 nm to 219.7 nm has been assigned to the peptide bond[134].

In order to compare UV spectra of diluted serum samples against known antibody samples, commercial rabbit IgG was obtained from Sigma. The antibody was developed in rabbits using highly purified proteins as immunogens, and the IgG fractions of the antisera were prepared by fractionation and ion-exchange chromatography. The product was delivered in lyophilized (freeze dried) form and had been evaluated by the producers for purity, specificity and potency using immunoelectrophoresis and quantitative precipitin analysis. (The latter is a general immunological technique which relies on precipitation of immune complexes as specific concentrations of antigen or antibody.) The product was stored in a refrigerator at 2 to 8°C.

The rabbit IgG was weighed out using a Mettler AE 200 four place analytical balance using tweezers for transferral of

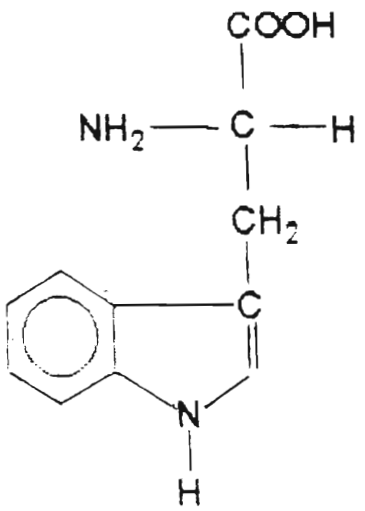


Figure 4.10. Structure of tryptophan.

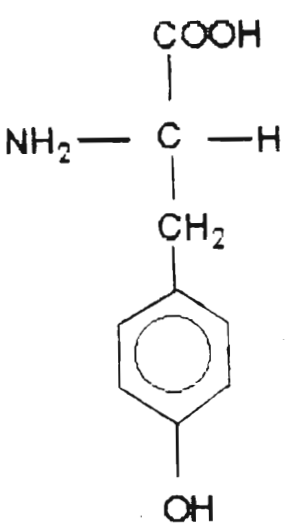


Figure 4.11. Structure of tyrosine.

antibody. A mass of 0.008 g of IgG was weighed out and transferred to a 1 ml aliquot of PBS contained in an Eppendorf vial. The antibody dissolved instantaneously when the vial was shaken by hand. Because of the small mass involved, the antibody was difficult to weigh out and it would thus have been logical to weigh out a larger mass of IgG and dilute the sample using PBS. However, at these higher concentrations, the antibody reached its solubility limit in PBS.

A UV absorbance spectrum of 0.8 mg/ml commercial rabbit IgG is seen in Figure 4.12. The accuracy in weighing out a small mass of IgG was tested by calculating the extinction coefficent of the IgG using the Beer-Lambert Law. A series of UV spectra were determined for a 0.4 mg/ml IgG concentration and 1 cm pathlength. The average extinction coefficient for these runs was found to be 1.43 $(mg/ml)^{-1}$ cm^{-1}. This value compares favourably with the value of 1.50 $(mg/ml)^{-1}$ cm^{-1} determined by Phillips and Kalthoff[135] and others[136]. Because the serum contains transferrins and albumins (serum proteins) as well as antibodies, antibody concentrations in the serum were not determined using the abovementioned Beer-Lambert Law and calculated extinction coefficient. As will be shown in Section 6.3, absolute values of antibody concentrations were not used in constructing a calibration graph relating antibody dilutions (determined by immunoblotting) to thymine dimer concentrations.

The following Sections (4.2.3.2 and 4.2.3.3) outline the initial purification technique (ammonium sulphate precipitation) of the crude serum that was employed in this research. First, the rationale of salt precipitation of proteins will be discussed. Then, the protocol which was used for antibody precipitation in this work will be discussed.

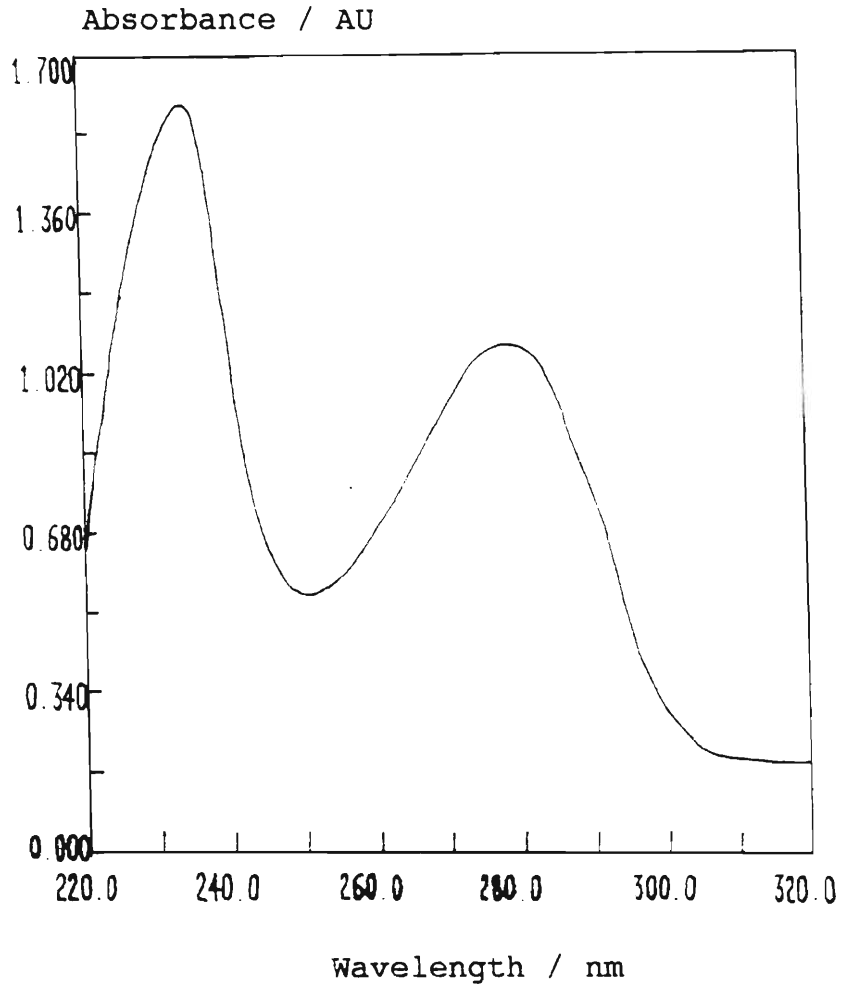


Figure 4.12. Absorption spectrum of 0.8 mg/ml commercial rabbit IgG.

4.2.3.2 Ammonium Sulphate Precipitation of Proteins: Explanation of the Technique

The purification of proteins is usually a formidable task; their fragile nature requires that careful control be exercised over factors such as pH and temperature because exposure to even moderate temperature and pH changes elicits slow denaturation. In addition, if the protein of interest is to be separated from other proteinaceous systems, very slight changes in iso-electric point (that pH or concentration where a protein is electrically neutral) or any other relevant criteria, must be invoked in order to effect separation and purification.

Because of the size and instability of proteins, the usual methods of purification of organic materials (distillation and solvent extraction) cannot be exploited. To complicate purification techniques even further, an evaluation of the degree of purification and overall yield must be performed for any particular purification regime. As an example, a procedure that results in a 100-fold purification of the protein, but yields only 1% of the product of interest is of little use.

Taking cognisance of the abovementioned factors, a number of fractionation techniques for protein purification are available. These include ion-exchange chromatography (where DEAE (diethyl-aminoethyl) cellulose anion-exchange columns or CM (carboxymethyl) cellulose cation-exchange columns yield sustantial purification of proteins[137]), electrophoresis (which gives excellent separations and permits near quantitative recovery of the protein of interest), molecular sieves, crystallization and affinity chromatography. This last mentioned technique will be discussed in detail in Sections 4.2.3.4 to 4.2.3.10.

Because of its ease of use, salt precipitation of proteins

was chosen as the initial purification technique in this work. First described in 1937, it is still the simplest and one of the most efficient techniques for protein purification, provided it is not necessary for the immunoglobulin classes to be separated from one another. The technique gives a preparation which contains all the immunoglobulins, other non-immune serum globulins and a low percentage of albumin[140]. Its advantages are the utilization of standard laboratory equipment at room temperature.

Proteins vary remarkably in their solubilities in concentrated salt solutions. Consequently, purification may be achieved by addition of sufficient salt to precipitate the protein of interest. This protein may then be collected by centrifugation. To this end, the salts most commonly used are ammononium sulphate and sodium sulphate. (Ammonium sulphate is usually preferred.) However, besides salts ethanol, dioxane and polyethylene glycol are also employed for this purpose[139].

Fractional separation by salt precipitation is thought to occur because the salt reduces the amount of free water surrounding the protein molecule as a hydration shell (Figure 4.13.). The solubility of the protein at any salt concentration is thus dependant on its own inherent ability to attract water molecules. With most proteins and biological materials, this is determined by electrostatic charge and the pH of the medium. The greater the difference between the pH and the pI (iso-electric point) of the protein, the greater the ability to form a water shell and hence, the less likely it is to precipitate.

Mahler and Cordes[138] quote ammonium sulphate precipitation as providing a 80% yield of the protein of interest with a 4-fold purification. As will be outlined in Section 6.1.1, this is in contrast to the approximately 90% recovery of

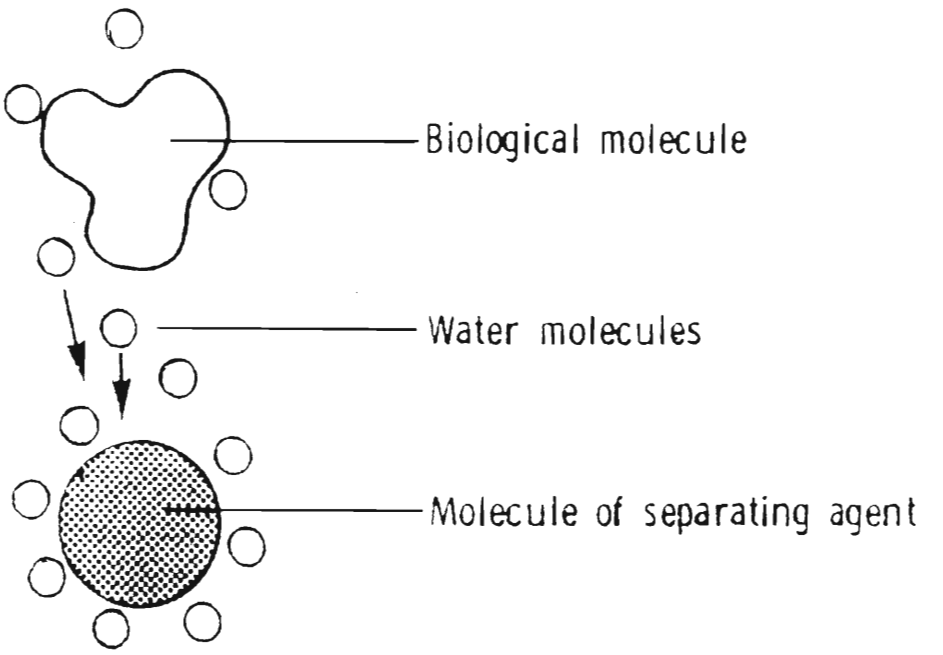


Figure 4.13. Proposed mode of action of salt precipitation of proteins.

IgG using affinity chromatography.

The following section describes the technique of ammonium sulphate precipitation of proteins, as employed for the purification of antibodies in this work.

4.2.3.3 Use of a Protocol for Ammonium Sulphate Precipitation of Antibodies

Although individual procedures for ammonium sulphate precipitation of proteins do vary, all procedures utilize saturated ammonium sulphate solutions and buffers. In this work, the procedure for ammonium sulphate precipitation of antibodies was supplied by G. Contrafatto of the Department of Biology at the University of Natal and used a saturated ammonium sulphate solution buffered at pH 6.5 for the precipitation of specific protein antibodies.

Merck analytical grade ammonium sulphate (132.14 g/mol) was used to prepare a saturated solution of ammonium sulphate according to the procedures of Dawson et al.[141] At 25°C, saturation is effected by the addition of 766.8 g of ammonium sulphate to sufficient water to make 1 dm^3 of solution. This mass was weighed out and transferred to a 1 litre "A" grade volumetric flask. Milli-Q water was added to bring the solution to the correct volume. This yielded a 4.06 M solution with a density of 1.2450 g/cm^3 as determined by Dawson et al[141].

A 0.5 M NaOH solution was prepared by weighing out 2.00 g of NaOH pellets (BDH, LAB grade, 40.00 g/mol), dissolving them in approximately 50 ml of Milli-Q water, transferring to a 100 ml volumetric flask and making up to the mark with Milli-Q water. Ammonia solution (BDH, LAB grade, 35%, 58.0 g/mol) was used to bring the saturated ammonium sulphate solution to pH 6.5. This was performed by adding 3.5% ammonia solution dropwise to 100 ml of the saturated

solution stirred on a magnetic stirrer. A portable Jenko pH meter calibrated against pH 7.0 and pH 4.0 UniVAR buffer solutions was used to determine the resulting pH which was between 6.45 and 6.5.

Because the volumes of serum which were obtained after centrifuging the rabbit blood were small (500 to 1000 μl), an even smaller aliquot of serum was used (200 to 500 μl) to purify the antibodies in the serum. A specific volume of serum was added to a 5 ml beaker and the serum was stirred using a magnetic stirrer. For a 500 μl aliquot of serum, 250 μl of saturated ammonium sulphate solution (pH 6.5) was added dropwise to the stirred solution using a 100 to 1000 μl Volac micropipette. Addition of the saturated solution turned the serum a milky colour. At this stage, the pH was carefully adjusted to 7.8 by the dropwise addition 0.5 M NaOH. A portable Jenko pH meter was used to determine the pH of the solution. Thereafter the solution continued to be stirred for 2 to 3 hours during which it turned to the clear colour it was prior to ammonium sulphate addition.

The solution was decanted into a 1.5 ml Eppendorf vial and spun for 2 x 15 minute sessions using the Hettich (12 000 rpm) Ultracentrifuge. A small white precipitate (protein) was noted at the base of the vial. The supernatant was decanted from the protein and this protein resuspended in the original volume (500 μl) of PBS. However, this was a tricky procedure which required the use a thin pointed capilliary tube to dislodge the white precipitate from the base of the vial and resuspend it in the buffer. The solution was transferred to a 5 ml glass beaker and the entire procedure of precipitation with ammonium sulphate repeated. Thereafter the solution continued to be stirred for a further 2 to 3 hours, was centrifuged in an Eppendorf vial and less protein was noted at the base of the vial in comparison to the amount noted the first time the precipitation was performed. The supernatant was decanted

and an attempt was made to dissolve the precipitate in 500 μl of PBS. Despite several attempts to dissolve the protein, it did not dissolve satisfactorily. Although this technique had apparently partly purified the serum components (by precipitation of the antibodies and some globulins[140]), the protein could still contain an extensive amount of ammonium sulphate which was then removed by the auxiliary procedure of dialysis against a 50 mM phosphate buffer (pH 6.8).

Preparation of the phosphate buffer involved the individual preparation of 50 mM Na_2HPO_4 (UniVAR) and 50 mM $NaH_2PO_4.2H_2O$ (BDH) by weighing out 7.0988 g of Na_2HPO_4 and 7.8009 g of $NaH_2PO_4.2H_2O$ respectively and making up to 1 litre in individual volumetric flasks. After filtering to remove particulate matter, 500 ml of each solution was mixed in a 1 litre volumetric flask, and the pH checked to be 6.8 using a portable Jenko pH meter.

Dialysis tubing (2.5 cm diameter) was prepared for use by boiling 10 to 15 cm strips in 1% Na_2CO_3 for 20 minutes, soaking in 10 mM EDTA for 20 minutes at room temperature, washing twice in Milli-Q water for 20 minutes each at room temperature and finally autoclaving in Milli-Q water.

The sample to be dialysed (500 μl) was transferred from the Eppendorf vial to the dialysis tubing (which was knotted at one end) with a micropipette. The other end of the tubing was knotted and the tubing immersed in approximately 50 ml of the phosphate buffer. The buffer solution was changed twice daily and the solutions dialysed for 4 days. Thereafter the dialysed and purified antibody solution was transferred with a micropipette to an Eppendorf vial.

Dialysis of this small volume of solution using standard dialysis tubing was incongruous as it led to numerous changes in concentration of the antibody during transferral

to and from the tubing. Such alterations would have been important if the purified serum was used in the immunoblotting protocol (Section 5.2.1.8) with enhanced chemiluminescence detection (Section 5.2.2.4). The situation was not improved when a smaller diameter tubing (0.60 cm) was employed, as the volumes being used were still too small. Despite the fact that ammonium sulphate precipitation can be used very successfully for the purification of large volumes of serum, and was used successfully for antibody purification in this work, the small volumes (500 μl) used dictated the use of an alternative purification technique.

This laboratory had just recently acquired a Waters Delta Prep 4000 HPLC system with a tunable absorbance 486 UV detector. It was decided to use this HPLC with a Memsep affinity chromatography cartridge to purify the crude serum.

Sections 4.2.3.4 to 4.2.3.10 describe the technique of affinity chromatography as well as the development and use of a working protocol for serum purification using this chromatographic technique.

4.2.3.4 Explanation of the Affinity Chromatography Technique

The separation of antibodies by chromatography has superseded ammonium sulphate precipitation of proteins as a separation technique. This has become necessary because of the requirement for pure antibody preparations in analytical, clinical and preparative biochemistry in which antibodies find application as components of immunological assay kits, as stationary phases in affinity chromatography and as carriers of therapeutic agents in cancer therapy. In turn, the need for advanced techniques to purify and analyse antibodies has been accelerated.

The chromatographic separation of antibodies relies on differences in four properties of the immunoglobulins: size and shape, charge, hydrophobic character and ligand specificity. These are all exploited in chromatographic separation regimes. Historically, purification of IgG has been achieved through a multistep process involving ammonium sulphate precipitation and anion-exchange chromatography on a DEAE cellulose column. IgG obtained from this two step process contains less than 10% of other proteins by mass[142]. A variation of this process is to use a chromatographic step in place of the salt precipitation. Here hydrophobic interaction chromatography coupled with anion-exchange chromatography has been shown to produce 95% pure IgG[143]. It has also been possible to couple chromatographic steps: a mixed-bed anion/cation-exchange column operated at low ionic strength near the pI of bovine IgG_1 has been used to produce IgG_1 of more than 95% purity from bovine serum[144]. IgG isolated by the procedures mentioned above is generally composed of one or more subclasses. Fractionation into sub-classes is most commonly achieved using ion-exchange chromatography and this separation technique was used for the resolution of mouse IgG_3 and IgG_{2b} from IgG_1 and IgG_{2a} which co-elute[145]. Hydroxylapatite absorbs biomolecules (nucleic acids and proteins) as a function of the extent and difference of their surface charge. This chromatographic technique (hydroxylapatite chromatography) has been used in the fractionation of both polyclonal and monoclonal antibodies[142]. A comparative study of different types of high performance chromatography indicates that hydroxylapatite chromatography is slightly superior to ion-exchange chromatography and hydrophobic interaction chromatography for the resolution of ascites monoclonal antibodies[146]. Hydroxylapatite columns are widely used in the purification of antibodies[147] giving more than 75% recovery. In addition, Juarez-Salinas et al.[148] have used high performance hydroxylapatite chromatography for the

separation of IgG molecules on the basis of idiotypes, (antigenic determinants characteristic of a particular variable domain of an immunoglobulin) even when the IgG molecules belonged to the same sub-group. Along the same lines, ion-exchange mediated separation is a specific regime relying on the immunoglobulin molecule having a pI more basic than any contaminating serum proteins[147]. In this way, the immunoglobulins subclasses (IgM, IgA, IgE, etc.) are separated from one another and can be collected in semi-preparative applications. However, as such specialized separations were not required, hydroxylapatite chromatography and ion-exchange chromatography were rejected as a purification technique in this work.

Separation of the immunoglobulins from contaminating serum proteins is more easily achieved using affinity chromatography, and to date, this has had limited application in antibody purification. With the advent of commercial affinity chromatography columns, the use of this technique is increasing and was chosen as the mode of separation in this work. The technique relies on the specific interaction of proteins (or other biomolecules) with ligands immobilized by covalent binding to an insoluble matrix. For example, an enzyme may interact with an immobilized substrate or an immobilized lectin may interact with the carbohydrate moeity of a glycoprotein. Elution of the adsorbed protein is carried out using specific eluants of pre-determined concentrations and pH's.

Affinity chromatography absorbents usually consist of three covalently linked components; the insoluble matrix, a spacer and the specific ligand. The insoluble matrix is normally composed of beaded 4% or 6% agarose which is stabilized by cross-linking with epichlorohydrin or bisoxirane. A cellulose matrix was used as the support medium for the Memsep 1000 affinity chromatography cartridge used in this work. Spacers are used as affinity

chromatography absorbents when a low molecular weight ligand is attached directly to the matrix. A hydrocarbon chain of 6 to 8 carbon atoms long is positioned between the matrix and the ligand and serves the function of reducing steric effects. When the ligand is a macromolecule (as in this work), the ligand is attached directly to the solid matrix and no spacer is used. Any molecule which interacts specifically with the protein, antibody or nucleic acid that is to be purified is potentially useful as a ligand in affinity chromatography. Enzyme substrates, lectins or antigens which interact with only a single type of protein are of this type. Others such as co-enzymes (NADH), nucleotides (ATP, AMP) and immobilized dyes interact specifically with a wider range of macromolecules.

An example of affinity chromatography related to UV-irradiation of DNA is given by Kronvall, Grey and Williams[149] who raised antibodies to UV-irradiated DNA and precipitated these antibodies using ammonium sulphate. The partly purified serum was applied to a 5 ml column of single stranded DNA-Sepharose for affinity adsorption of anti-single stranded DNA antibodies. After elution, the non-adsorbed material was applied to a 5 ml column of UV-irradiated single stranded DNA-Sepharose. Antibodies against UV-irradiated single stranded DNA bound strongly and eluted with a 4 M urea, 0.5 M NaCl and 0.2 M acetic acid pH 3.1 buffer. These purified antibodies were used in UV repair studies of UV-irradiated human skin.

Returning to the theoretical aspects of affinity chromatography, most ligands (usually proteins) can be immobilized on an insoluble matrix by reaction under mild conditions with agarose activated cyanogen bromide[150]. Included here is the Protein A molecule which has been shown to interact specifically with only the IgG immunoglobulin of selected mammals. Protein A is that cell wall protein (42 000 d) expressed by the bacterial species

Staphylococcus aureus which precipitates normal human gamma globulin as well as mouse, rabbit and guinea pig globulins. It has been stated that all humans have so called natural antibodies to this cell wall protein and it has been shown that the ability of normal human sera to agglutinate *S. aureus* is based on a reaction with this antigen which was subsequently named Protein A. Detailed studies on precipitin reactions have revealed that Protein A reacts with the mouse gamma 2_a, gamma 2_b and gamma 3 globulins as well as human IgG-1, -2 and -4 subgroups[151], while human IgD and IgE showed no reaction. Sub-group precipitation with rabbit sera has, to date, not been performed. However, for the abovementioned species, it is proposed that the last 19 amino acids of the heavy chain of gamma G globulins from horse, rabbit and humans are the same, which could partly explain the selectivity of reaction of Protein A[151].

The reaction between Protein A and IgG is a rather specific one. The distinct sub-group pattern as well as the reactivity of immunoglobulin sub-fragments indicate that the very specific structures of immunoglobulin chains are involved. Kinetic studies on the interaction between Protein A and human gamma globulins yielded values for this equilibrium constant of approximately 4×10^7 litres/mol[152]. This value far exceeds that of hapten-antibody reactions and is indicative of the high specificity of the reaction.

This Protein A-IgG reaction is postulated to be mediated by the F_c fragment of antibody molecules, unlike normal antigen/antibody reactions which involve the F_{ab} fragment of the antibody (see Section 1.2). For this reason, Protein A reactions with IgG are referred to as "pseudo-immune" reactions. Langone[153] has shown that the binding of the Protein A molecule to the F_c region of the antibody molecule does not affect the antibody ability to bind antigen. In

addition, Langone et al.[154] have postulated that 4 tyrosine residues of a Protein A molecule are responsible for its ability to bind the F_c domain of IgG. Mota, Ghetie and Sjoquist[155] have proposed that complex formation between IgG and Protein A in rabbits involves two IgG molecules binding to a single Protein A molecule. This hypothetical model is illustrated in Figure 4.14.

Apart from purifying sera in biochemical and analytical applications, formalinized Protein A containing staphylococci have been used as particulate adsorbents. Lind and Mansa[156] adsorbed 93% of rabbit IgG from a serum sample using this technique. The adsorption efficiency correlates well with the 90% recovery of commercial rabbit IgG using the Memsep cartridge in this work (see Section 6.1.1).

Other applications of Protein A include labelling Protein A with ^{125}I, followed by immobilizing an antigen to a solid bead support and incubating the antigen with the Protein A-^{125}I complex. Scintillation counting of the resultant radioactivity allows the antigen to be quantitated. Under optimal conditions, research of this nature allowed picomole levels of the antigen to be determined. In this manner, Langone et al.[154] determined the fluid phase IgG concentrations in human and rabbit sera to be 9.5 and 13.6 mg/ml respectively.

If only the antibodies generated against thymine dimers were required, an affinity column could have been packed with commercially synthesized oligo-dT. The relevant antibodies would bind to the oligo-dT, while the non-thymine dimer antibodies would elute from the column. However, such a purification step was prohibitively expensive considering both the cost of the oligo-dT and the specialized knowledge and expertise required to pack an affinity column. Extending quantitation of thymine dimers

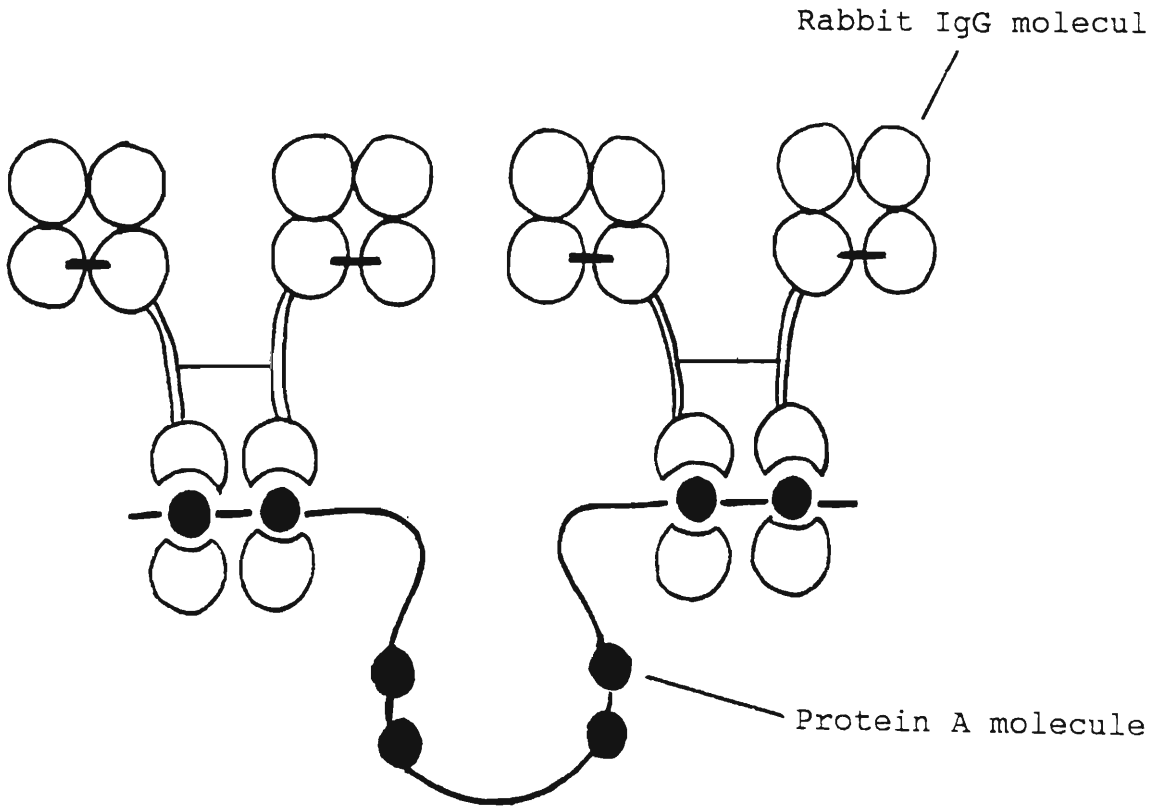


Figure 4.14. Hypothetical model of Protein A-rabbit IgG binding.

to cellular systems in this laboratory might well require oligo-dT affinity purified antibodies for accurate quantitation, and in this instance an oligo-dT packed affinity column would be required.

Affinity chromatography thus separates immunoglobulins from other serum proteins and hence effects separation. However, Protein A only binds to the sub-classes of the IgG molecule, allowing the IgM, IgA and IgE antibodies to pass straight through. Because IgG comprises between 75 and 80% of all immunoglobulins and because antibody purification was not used in any quantitative work in this research, it was adequate for this percentage of the antibody population to be purified. If quantitation of antibodies themselves was important, then affinity chromatography is limited as it is unable to separate immunglobulin sub-classes from each other.

The following sections (4.2.3.5 and 4.2.3.6) describe the equipment (Memsep Chromatography 1000 affinity chromatography cartridge attached to a Delta Prep 4000 Preparative HPLC system with UV detection) that was used in this work for the purification of rabbit IgG from other rabbit immunoglobulins and serum proteins.

4.2.3.5 Affinity Chromatography Cartridge Operation

In this work the Protein A ligand was housed in a Memsep Chromatography 1000 affinity chromatography cartridge supplied by Millipore. The cartridge has been designed for the rapid extraction, concentration and purification of immunoglobulins from complex feed streams such as tissue culture media, ascites fluid or serum. Other applications include the separation of plasma proteins, extracellular proteins, inositol phosphates and the separation of nucleic acids from proteins and nucleotides. To this end, the matrix and ligand are housed within a polypropylene capsule

containing mesh distributors (Figure 4.15.). The matrix consists of 1.2 μm regenerated cellulose disc membranes positioned on top of one another (Figure 4.15.). Diffusion problems are eliminated by the pore size used which allows even the largest of biomolecules easy access to all available active sites. Thin diffusion channels at each end of the membrane stack ensure efficient flow distribution while minimizing the dead volume of the unit. In addition, the regenerated cellulose network excludes problems such as bed shrinkage at high saline concentrations or bed movement. Air bubbles trapped in the unit are easily removed by increasing the flow rate of the mobile phase. Memsep cartridges are supplied with a variety of active groups depending on the specific application of the cartridge. DEAE, carboxymethyl, quaternary methylamine or Protein A are incorporated into Memsep cartridges. The Memsep 1000 has a 1.4 ml bed volume, while its larger counterpart (Memsep 1010) has a 4.9 ml bed volume. The smaller cartridge was chosen in this work because of the cost factor involved.

These cartridges have a pH operation range of 2 to 11 which makes them suitable for the purification of the wide variety of biological fluids mentioned above. Temperatures of 50°C were not exceeded in this work, and it was imperative that an operating pressure of 100 psi (6.87×10^5 Pa) or less be used for fear of the cartridge exploding. This consideration implied that mobile phase flow rates had to be maintained between 1.5 ml/min to 8.0 ml/min. However, in this work, flow rates greater than 1.5 ml/min did cause backpressure build up, presumably due to buffer precipitation in the solvent lines. Hence, flow rates greater than 1.5 ml/min were not used in this work.

The advantages of using Memsep cartridges for purification are manifold. The pure cellulose matrix exhibits very low non-specific protein binding and hence immunoglobulin

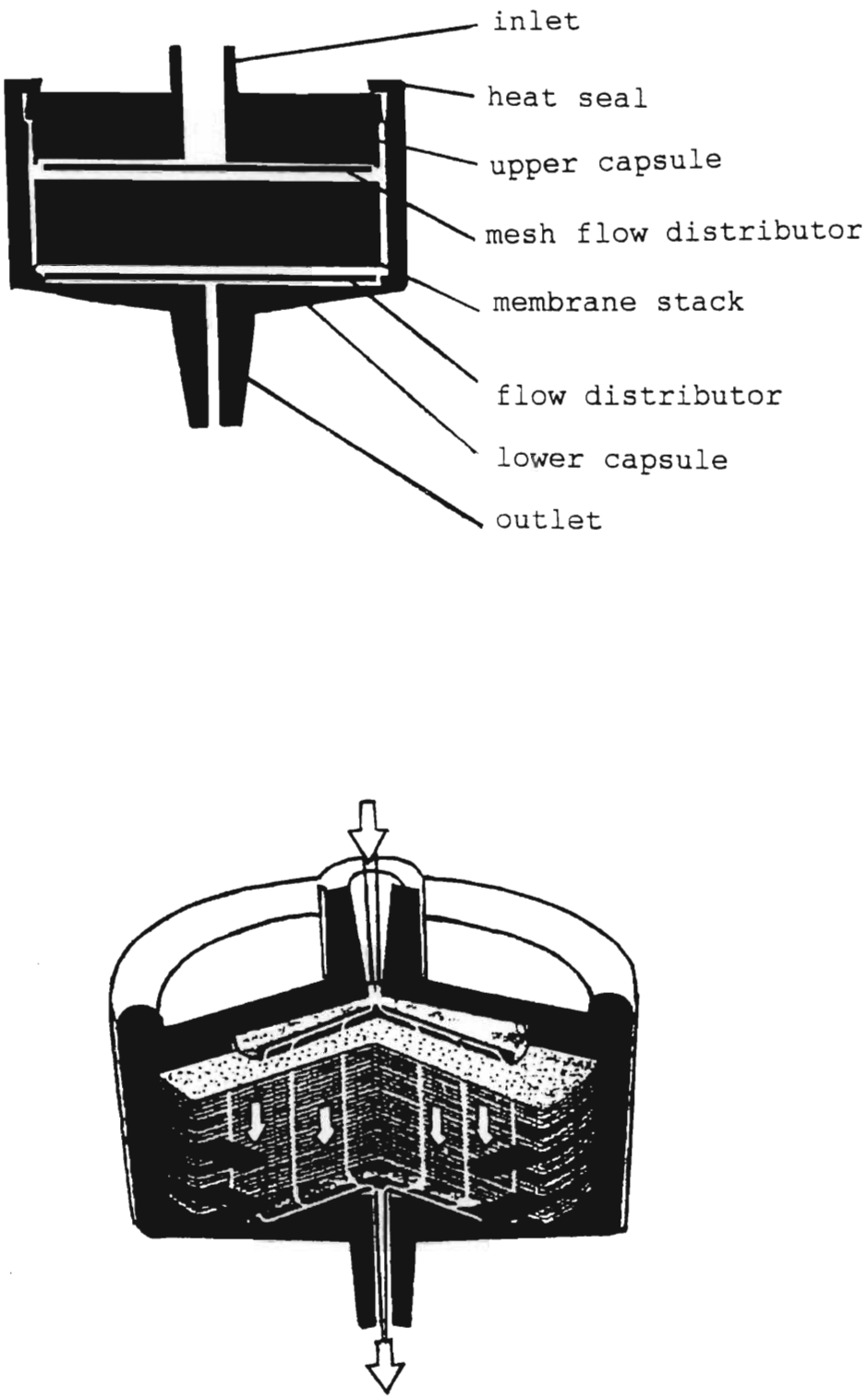


Figure 4.15. Schematic representations of the Memsep 1000 Affinity Chromatography Cartridge.

recovery is maximized. Control of functional group incorporation in the manufacture of the cartridge ensures that reproducible performance is obtained. Memsep cartridges can be re-equilibrated and regenerated up to 100 times for use. Finally, the cartridge is compatible with syringes and peristaltic pumps and can easily be attached to conventional HPLC equipment.

This attachment to an HPLC system is effected by a series of threaded connections and ferrules (Figure 4.16.) attaching the inlet of the cartridge to the injector valve and the outlet to a detector or fraction collector.

Seeing that the operation of the Delta Prep 4000 Preparative HPLC has not been discussed by any members of this research group, the following section (4.2.3.6) discusses the workings and operation of this HPLC.

4.2.3.6 Preparative HPLC Equipment and Operation

The HPLC system used in this study is a Waters Delta Prep 4000 HPLC solvent delivery system that can be used for qualitative, analytical and preparative scale chromatography. It was attached to a Waters Tunable 486 Absorbance UV detector and Waters 745 Data Module with integration functions. The HPLC system accommodates large scale and small scale operation without replumbing the system, conveniently allowing direct scale-up of samples. Figure 4.17. illustrates the Delta Prep system. The controller (1) houses the electronics of the system, thereby controlling solvent gradients, flow rates and sparging during analysis. The fluid handling unit (2) consists of the pump heads with two 500 μl volume pump heads, the solvent sparge valve assembly and solvent proportioning valve assembly. The column rack (3) contains the rheodyne injector, pump purge valve, column switching valve and stainless steel tubing arrangements for

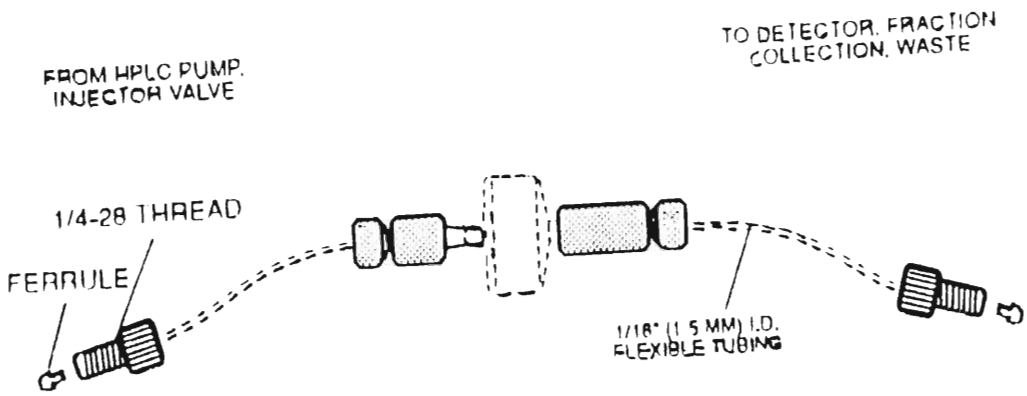


Figure 4.16. Connections for the attachment of the Memsep cartridge to the HPLC.

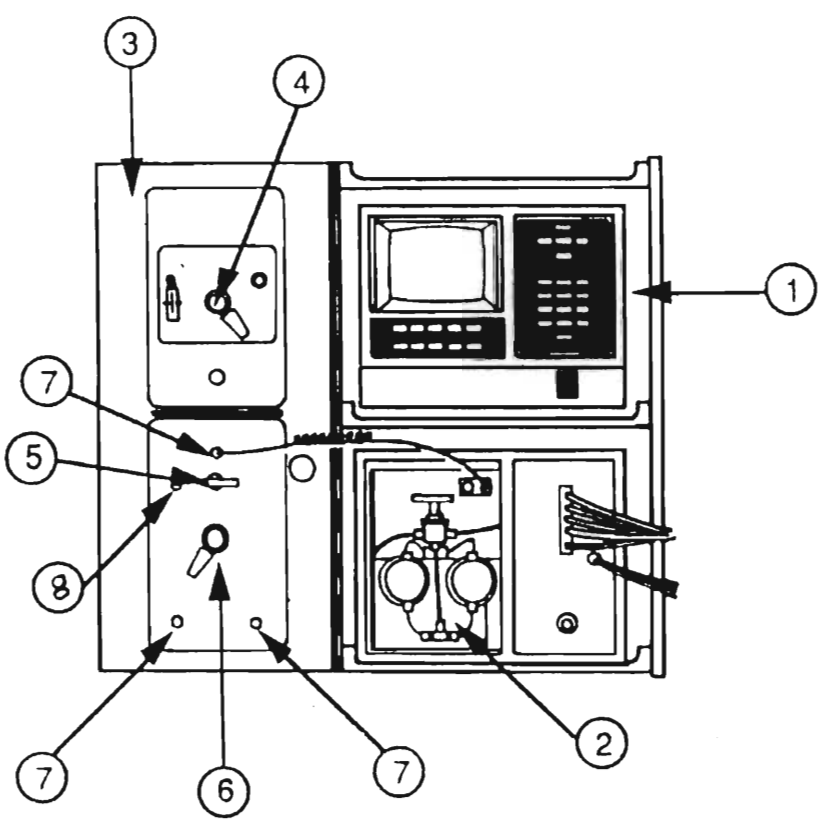


Figure 4.17. Schematic representation of the Waters Delta Prep 4000 HPLC system.

analytical or preparative columns. The Rheodyne 7010 injector (4) contains fixed sample loops allowing a constant volume of sample to be injected each time. In this work, a 100 μl fixed volume loop was attached to the injector replacing the 5.1 ml loops the system was supplied with (see Section 4.2.3.8.3). The purge valve (5) allows a choice of solvent flow; either from the pump to waste, or through the injector, columns and rest of the system. The column switching valve (6) allows either the small scale or large scale tubing arrangements to be chosen, while the inlet and outlet ports (7) accept the outlet from the pump for either the small scale or large scale arrangements. The purge outlet port (9) directs solvent flow from the inlet port to this outlet port when the purge valve selects the purge mode.

The Waters tunable 486 absorbance detector is a single channel tunable UV/Vis detector designed for HPLC applications. The detector can operate as a stand alone unit (when attached to a chart recorder) or with other system modules such as the Waters 4000 delivery system and integrator. It provides a usable light intensity over the 190 to 600 nm range, although it is usual for only the UV range (190 to 400 nm) to be used.

The optical design of the 486 detector includes a deuterium source lamp, illumination lens, aperture slit, diffraction grating, beam splitter, flow cell (sample and reference sides) as well as a dual photodiode. Figure 4.18. illustrates the optics showing the path of a light beam as it passes through the components in the optics assembly. The dual light beams pass through the sample and reference sides of the flow cell to dual photodiodes where they are converted to analogue currents. A pre-amp board serves to convert this current to digital signals for processing by the central processing unit and output to a chart recorder, integrator or computer.

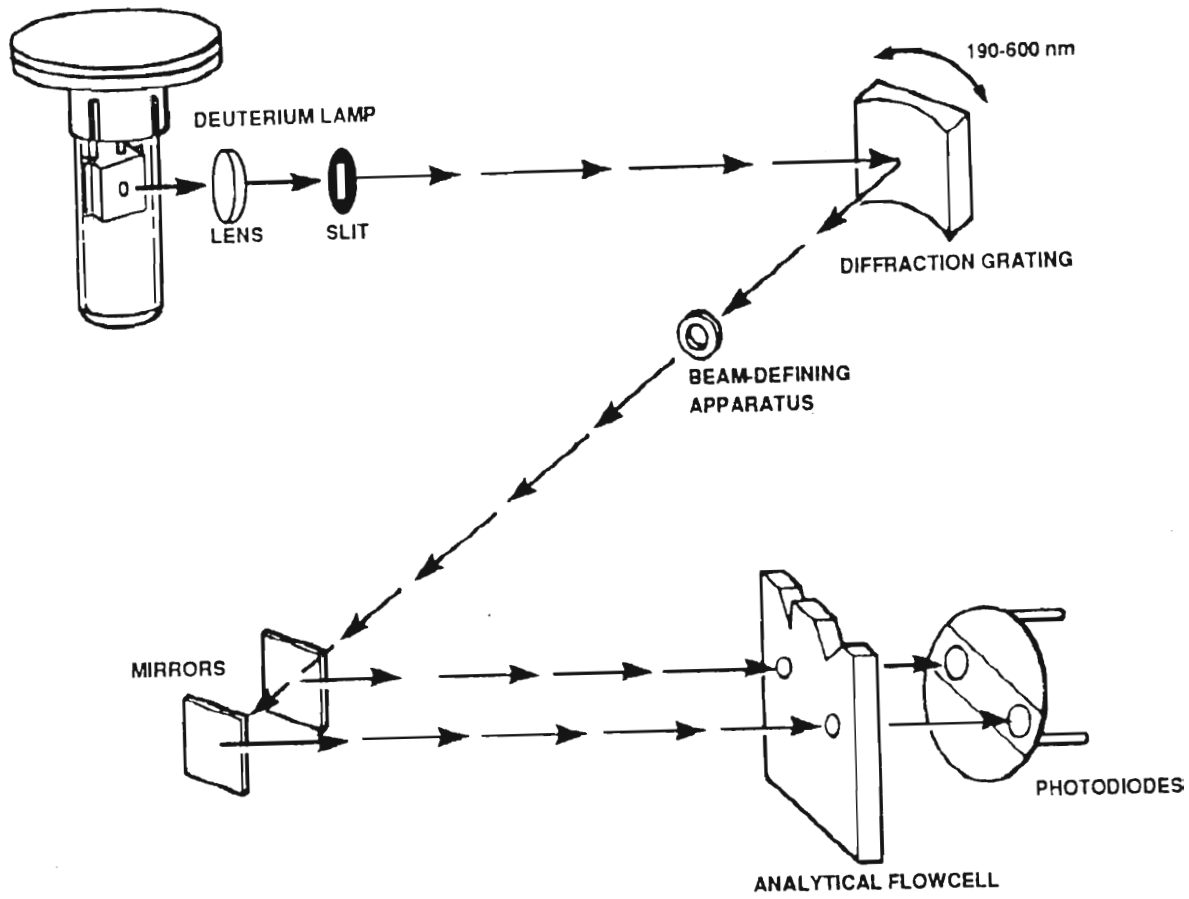


Figure 4.18. Schematic representation of the path of a light beam through the optical configuration of the Waters 486 detector.

The flowcell contains the Taper-cell designed to eliminate common absorbance detection problems. These include refractive index changes due to changes in solvent. In this case stray light bends within the flow cell causing absorbance errors. Gradient changes may cause refraction as well as reflection at the junction of the window and cell. The patented Taper-cell corrects these unwanted phenomena permitting true absorbance detection to occur. The analytical cell has a 10 mm path length while in the preparative cell the path length is reduced to 3 mm.

During a 1 hour lamp warm up, wavelength calibration of the instrument occurs automatically. The detector locates and identifies the 656 nm peak in the deuterium lamp emmision spectrum to ensure that the calibration is correct.

On the electronics side, the 486 detector design is digitally based and includes hardware such as the front panel keyboard, printed circuit boards and their interconnections. The detector software includes features such as sensitivity and filter settings, wavelength selection, chart polarity and lamp on/off control.

The Waters 745 data module uses a three function keyboard to receive, process and store digital information from the detector. Function keys allow operations such as peak width, peak threshold, attenuation and chart speed to be altered for specific requirements during operation. Sample tables can be set up in memory allowing reports to be generated of component name and concentration, retention time, peak area and detector response factor. In this manner, a comprehensive list of relevant data is obtained after an injection.

The following section describes the solvent and sample preparation procedures that were employed in this work for serum purification using the Memsep 1000 affinity

chromatography cartridge.

4.2.3.7 Preparations Required Before Use of the Memsep Cartridge

This section describes the preparation of the buffer solutions (0.05 M Tris/HCl, 0.1 M NaCl, pH 8 and 0.1 M glycine/HCl pH 2.5 and 0.02% sodium azide in 0.1 M Tris/HCl pH 8) and samples. The attachment of the Memsep 1000 cartridge to the Waters 4000 Delta Prep HPLC is also discussed. The development of a working protocol for serum purification using the Memsep cartridge will be discussed in Section 4.2.3.8.

4.2.3.7.1 Preparation of Buffer Solutions

The buffer solutions used in this work were those recommended by Millipore. A 0.05 M Tris/HCl, 0.1 M NaCl, pH 8 (Tris/HCl buffer) was used for sample loading and equilibration. It was prepared by weighing out 6.0575 g of Tris (Tris[hydroxymethyl] aminomethane; Saarchem analytical grade, 121.14 g/mol) and 5.8442 g NaCl (Saarchem analytical grade, 58.44 g/mol). The salts were dissolved in a beaker containing approximately 500 ml Milli-Q water and stirred magnetically. At 25°C, a pH of 8.0 was established by the addition of 292 ml 0.1 M HCl[141] to the salt solution. Acid addition was performed using a 100 ml burette. The solution was transferred to a 1 litre volumetric flask and made up to the mark using Milli-Q water. The pH of the solution was checked using a Jenko portable pH meter and in all cases, the pH was between 7.95 and 8.05. As mentioned in Section 4.2.3.3, proteins are particularly susceptible to pH changes. Hence buffer preparation was routinely carried out with great care.

A 0.1 M glycine/HCl, pH 2.5 solution (glycine/HCl buffer) was used as the IgG elution buffer in this work. A mass of

7.507 g of analytical grade glycine (BDH, 75.07 g/mol) was added to a 1 litre volumetric flask and Milli-Q water was added to dissolve the glycine and make the solution made up to 1 litre. Then, 250 ml of this solution was added to 142 ml of 0.1 M HCl[141] in a 1 litre volumetric flask. Milli-Q water was used to bring the solution to the correct volume. The pH was checked to be between 2.45 and 2.50 in all cases using a Jenko portable pH meter. Because the glycine was an easily available source of food, this solution was particularly susceptible to microbial attack. To inhibit degradation of this solution, it was stored in a refrigerator when not in use.

The 0.02% sodium azide in 0.1 M Tris/HCl, pH 8 was used as the storage buffer for the Memsep cartridge when the cartridge was not in use. Sodium azide has recognized anti-bacterial properties, but care was taken in solution preparation because of its potentially explosive properties. A 0.1 M Tris solution was prepared and 0.1 M HCl added to establish a pH of 8. Then, 0.02 g of sodium azide was carefully weighed out and transferred to a 100 ml aliquot of this Tris/HCl solution.

All buffers were filtered through 0.45 μm Durapore filters to remove insoluble material. Apart from blocking the solvent lines, optically dirty buffers and solvents cause baseline noise and drift, and columns become blocked. Filtration also degassed the buffers. Degassing served the important function of eliminating oxygen from solution, thereby preventing oxidation of the buffers themselves. In HPLC applications, oxygen is known to increase noise because less light reaches the detector thus causing baseline drift. At pH 8, a 0.2 M Tris/HCl solution has a UV cut-off of 212 nm. At pH 8, a UV spectrum of the 0.05 M Tris/HCl, 0.1 M NaCl showed a 214 nm cut-off which did not affect the 280 nm detection wavelength used in this work.

4.2.3.7.2 Preparation of Serum Samples

Millipore suggested dilution of serum samples prior to filtration of these samples. In this work, 1:10 or 1:50 dilutions were performed which minimized clogging of the filtration apparatus. Filtration was performed because biological samples are often contaminated with particulate and bacteriological matter which, if not removed, leads to premature clogging of the Memsep cartridge itself.

Sample preparation by means of filtration was performed using Millex HV filtration units which contained 0.45 μm Durapore polyvinylidene as the filters. These filters are hydrophobic and have low protein binding characteristics. They are contained in a PVC housing (Figure 4.19.). A single unit was attached to a 2.5 ml hypodermic syringe (without a needle) containing the diluted serum sample. By depressing the plunger, the sample was filtered through the unit. The filter was used until the filtration membrane was clogged with particulate matter. Once filtered through 0.45 μm filters, the serum samples were filtered through Millex GV filtration units which contained 0.22 μm Durapore polyvinylidene filter units. This product removed all micro-organisms and particulate matter and precipitated dissolved powders greater than 0.22 μm in size. The Millex GV units were supplied sterile and were only to be used once. However, sterile conditions were not important during HPLC analysis and hence the filter was re-used until it became clogged. For both the Millex HV and GV filters, a pressure greater than 75 psi was avoided for fear of the Durapore membrane disintegrating.

4.2.3.7.3 Attachment of the Memsep Cartridge to the Preparative HPLC

As mentioned in Section 4.2.3.5, the Memsep cartridge can be attached to a peristaltic pump system as well as an

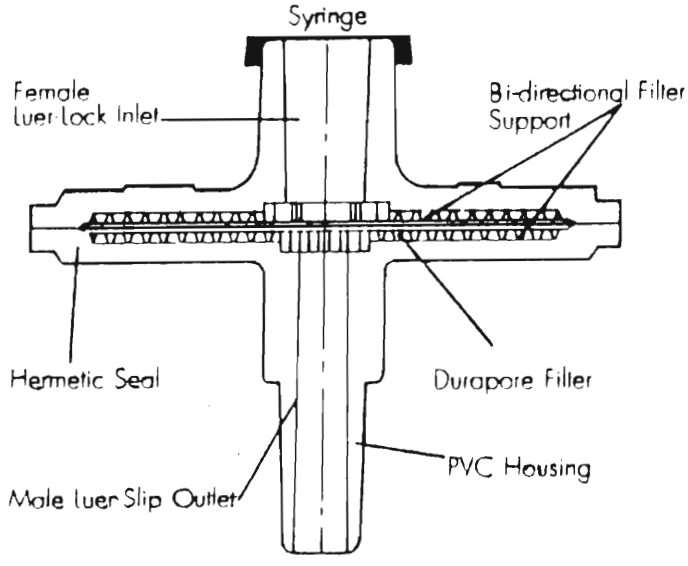


Figure 4.19. Schematic diagram of the Durapore polyvinylidene filtration unit.

HPLC. In this work, using the small-scale set up, it was easy to insert the cartridge between the injector and detector using the threaded connections and tubing mentioned in Section 4.2.3.5. Initially 9" stainless steel tubing (actually 1/9000 of an inch tubing, but referred to as 9" tubing by the manufacturer) was used at the inlet and outlet of the rheodyne injector. When backpressure problems were experienced, this tubing was replaced with 20" tubing which meant the samples were easier to inject. However, a larger diameter tubing caused turbulent flow of the sample within the tubing, resulting in broadening of the IgG fraction on the chromatogram. Once the back pressure problems were solved (by setting the mobile phase flow rate at 1.5 ml/min), the tubing was replaced with 9" tubing.

The serum sample was injected manually into the rheodyne injector; it passed directly to the Memsep cartridge before moving to the analytical flow cell of the UV detector. Because only 100 μl of sample was accommodated on the loop, the remainder went to waste where it could be re-injected if required.

4.2.3.8 Development and Use of a Working Protocol for Serum Purification Using Affinity Chromatography

Solvent and sample preparation as well as the attachment of the Memsep cartridge to the HPLC was described in the previous section. Now, the development and use of a working protocol for serum purification will be discussed in detail.

4.2.3.8.1 Development of a Working Protocol

Once attached to the HPLC, the Memsep cartridge was equilibrated using Tris/HCl buffer before use. Freshly filtered buffers were prepared, and the Memsep cartridge was equilibrated by passing more than 20 column volumes of

Tris/HCl through the device. A PT EVAL (peak threshold evaluation) of between 12 and 200 delivered by the attached data module indicated that the instrument was sufficiently stable to use.

When a run was performed, a lamp warm-up of 1 hour was necessary. As outlined in Section 4.2.3.6, wavelength calibration took place automatically during this time. The detection wavelength was set at 280 nm for maximum protein absorbance. First, a blank injection was made to ensure a flat baseline. The latter demonstrated that the buffers were not contaminated so as to cause a measurable absorbance to be registered by the detector. The diluted serum sample was then loaded onto the syringe and manually injected through the rheodyne injector onto the Memsep cartridge. The Memsep cartridge was rinsed with the Tris/HCl buffer until the absorbance of the eluant at 280 nm returned to zero. As mentioned previously, the Tris/HCl buffer was used to load the serum sample and as will be outlined in Section 4.2.3.10, it eluted the serum proteins as well as other immunoglobulins whilst simultaneously adsorbing the IgG onto the cartridge. Desorption of the antibody was performed by passing the glycine/HCl buffer through the cartridge and monitoring the absorbance at 280 nm until it returned to baseline. The eluate was collected as fractions containing the IgG for further analysis. If the cartridge was not to be re-used immediately, more than 20 column volumes of 0.02% sodium azide in 0.1 M Tris/HCl was passed through it. The cartridge was detached from the HPLC, re-capped and stored in a refrigerator until required. If the cartridge was to be re-used immediately, the Tris/HCl buffer was used to re-equilibrate it.

The step-by-step workings of the Memsep cartridge were described above. However, the entire protocol was optimized in terms of the gradients and flow rates used, as well as optimizing the integrator functions and sensitivity. The

following sections describe this optimization process.

4.2.3.8.2 Optimization of Flow Rates and Gradients

As mentioned in Section 4.2.3.4, the upper threshold pressure limit of the Delta Prep 4000 was set at 100 psi for fear of the Memsep cartridge exploding at backpressures higher than this. This implied that flow rates greater than 8 ml/min were not to be exceeded in this work. However, despite extensive rinsing of the solvent lines using filtered Milli-Q water, crystallization of buffers was still occuring to a small extent in the lines, and did cause excess backpressures to develop. Hence, flow rates less than 8 ml/min were used in this work.

From a chromatographic point of view, the effect of flow rate on sample resolution was investigated. With other parameters (gradients, sample dilution, attenuation) held constant, a 1:50 dilution of a serum sample was injected onto the Memsep cartridge using 1.0, 1.5 and 2.0 ml/min buffer (Tris/HCl and Glycine/HCl) flow rates. Figures 4.20., 4.21. and 4.22. illustrate the resulting chromatograms. Although much noise is noted on the 1.5 and 2.0 ml/min chromatograms, these flow rates do allow the IgG component to elute earlier (18.3 to 21.7 minutes for the 2.0 ml/min, as opposed to 34.6 to 39.7 minutes for the 1.0 ml/min sample). This consideration is important if multiple samples need to be run within a short space of time. Because of the backpressure and crystallization problems mentioned earlier, a flow rate of 1.5 ml/min was chosen for this work.

However, to reduce the elution time of the component of interest (and hence speed up analysis), another parameter can be varied. Figures 4.23. and 4.24. illustrate separation using step gradients pre-programmed into the Delta Prep 4000 solvent delivery system. In Figures 4.20 to

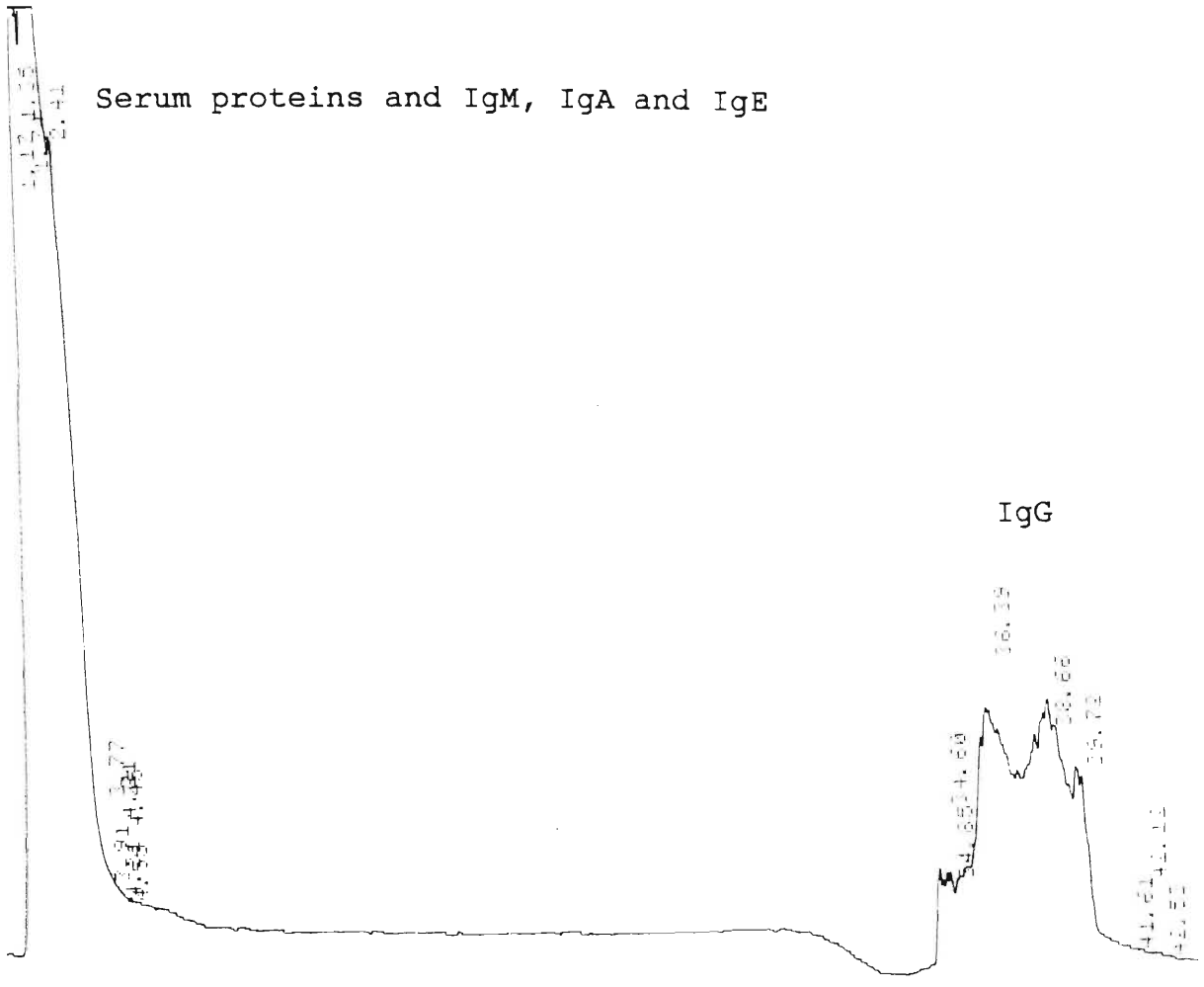


Figure 4.20. HPLC chromatogram illustrating separation of the IgG fraction from the serum proteins and other immunoglobulins using a 1.0 ml/min flow rate of the Tris/HCl and Glycine/HCl buffers.

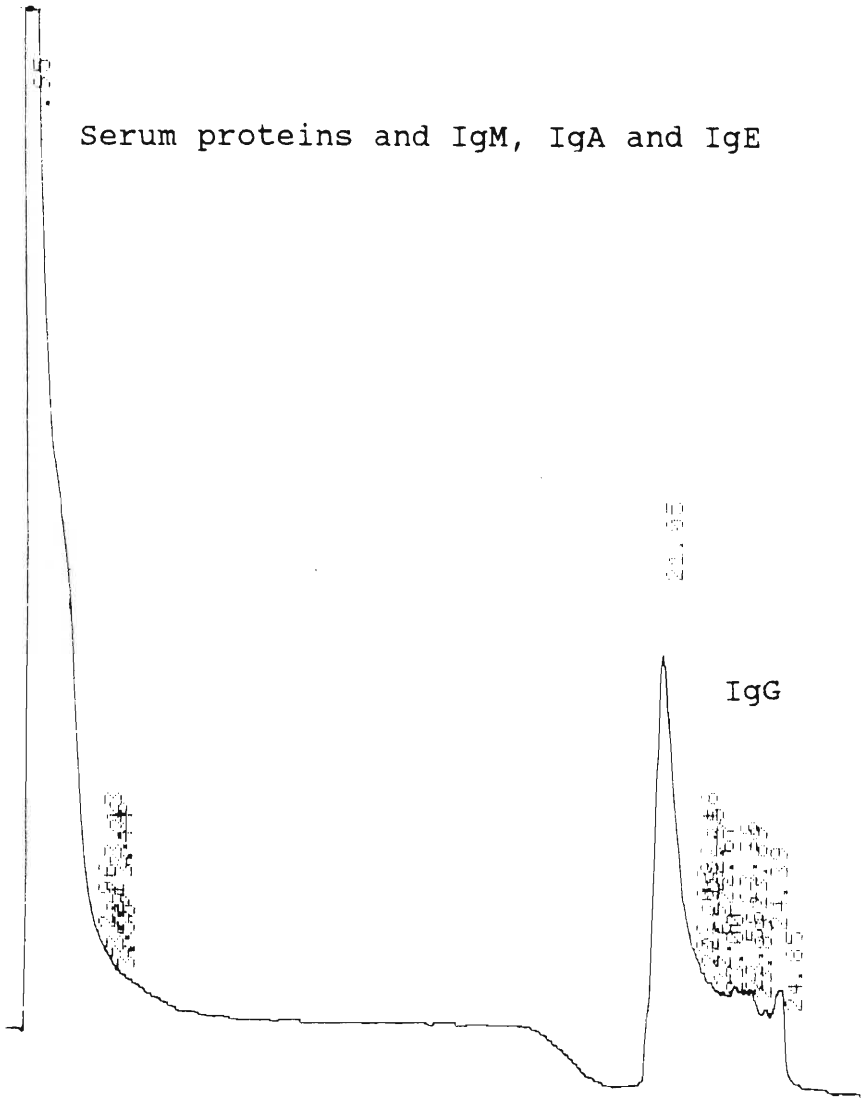


Figure 4.21. HPLC chromatogram illustrating separation of the IgG fraction from the serum proteins and other immunoglobulins using a 1.5 ml/min flow rate of Tris/HCl and Glycine/HCl buffers.

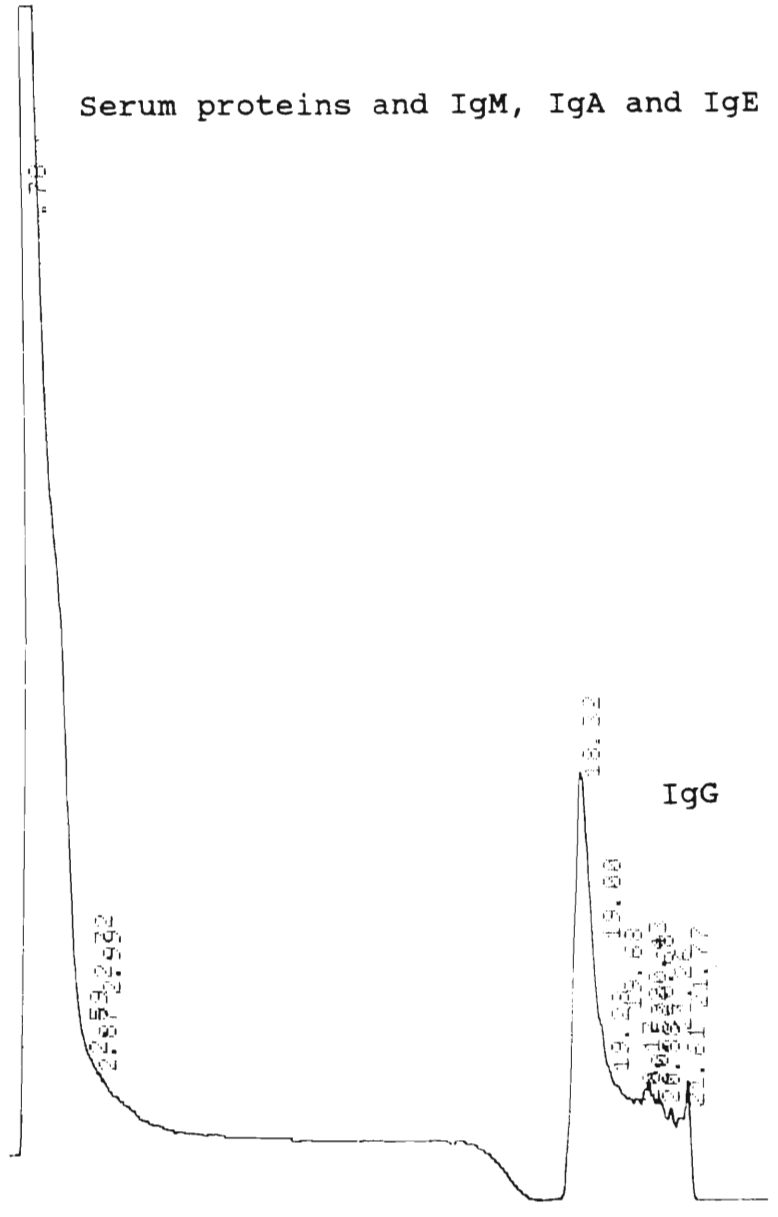


Figure 4.22. HPLC chromatogram illustrating separation of the IgG fraction from the serum proteins and other immunoglobulins using a 2.0 ml/min flow rate of Tris/HCl and Glycine/HCl buffers.

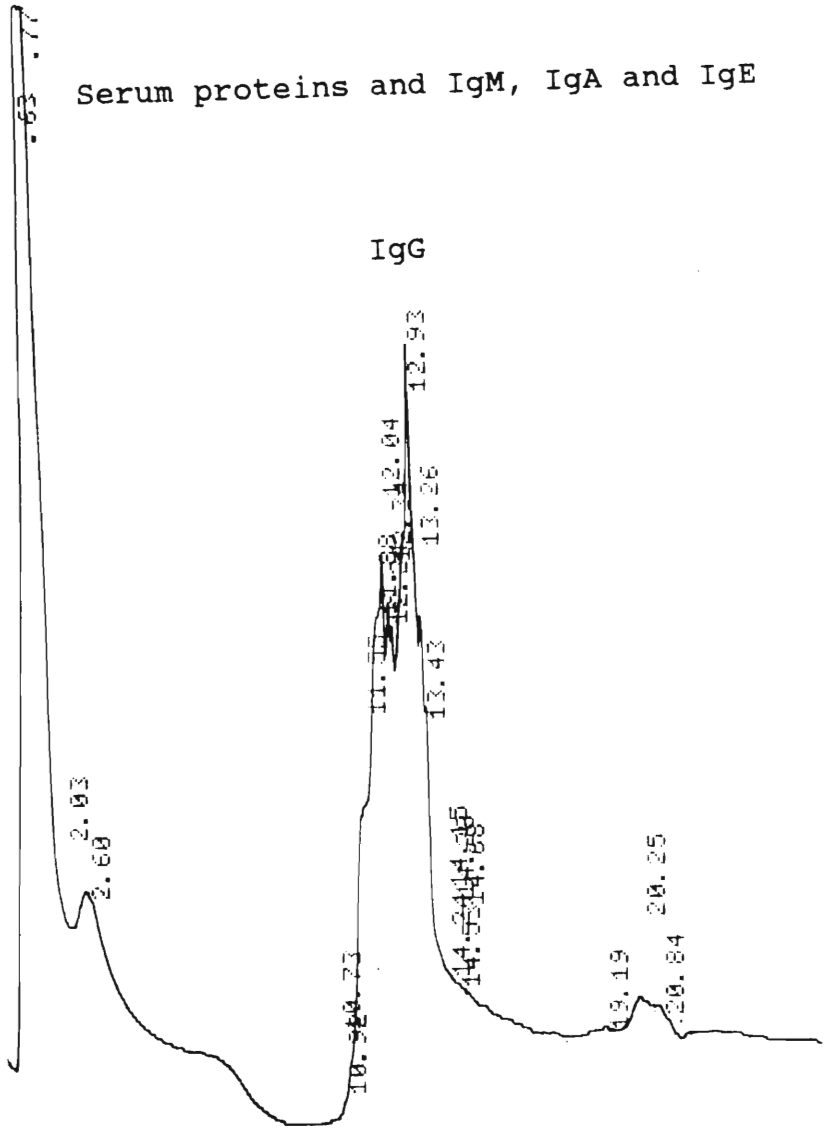


Figure 4.23. HPLC chromatogram illustrating separation of the IgG fraction from the serum proteins and other immunoglobulins with the Tris/HCl buffer loading the serum sample for 0 to 15 minutes.

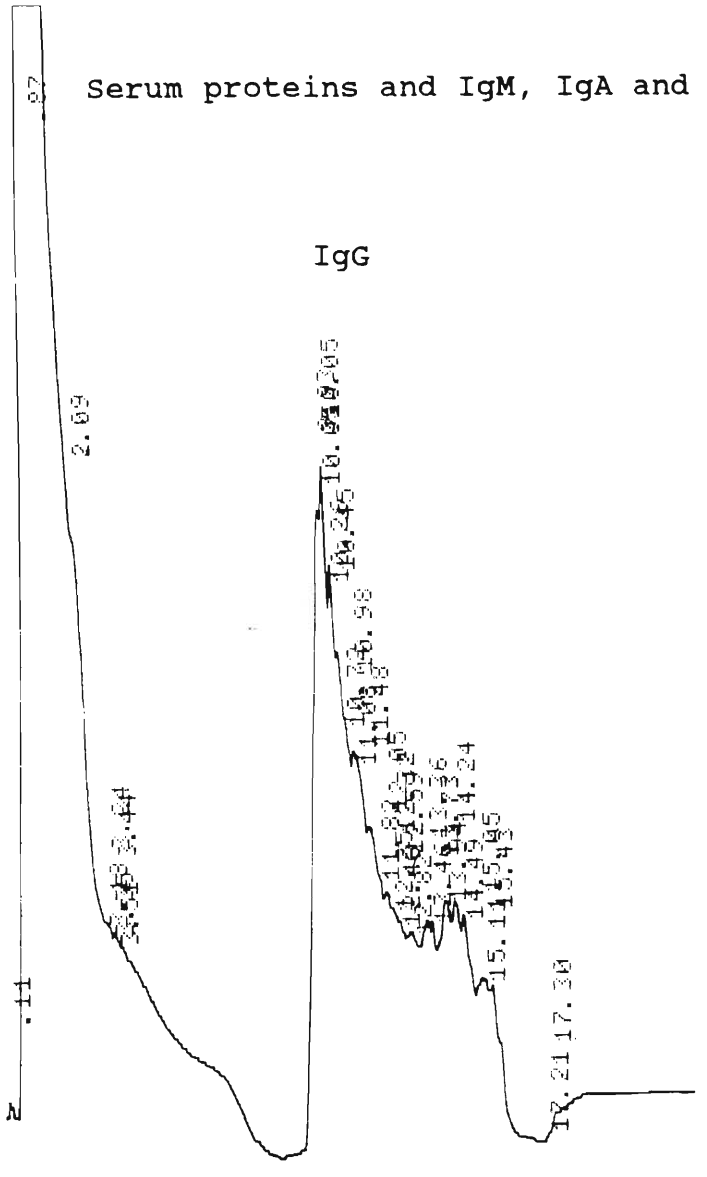


Figure 4.24. HPLC chromatogram illustrating separation of the IgG fraction from the serum proteins and other immunoglobulins with the Tris/HCl buffer loading the serum sample for 0 to 12 minutes.

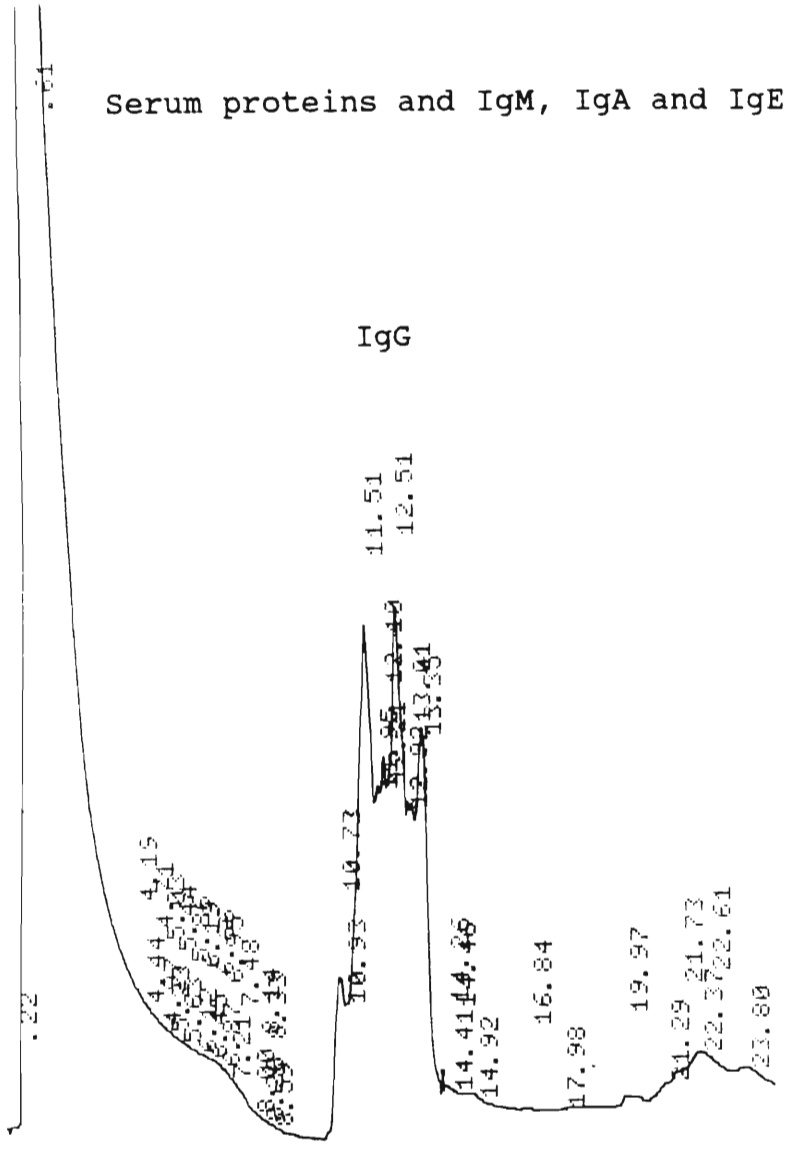


Figure 4.25. HPLC chromatogram illustrating separation of the IgG fraction from the serum proteins and other immunoglobulins using a 1.5 ml/min flow rate of buffers, with Tris/HCl loading the serum sample for 0 to 2 minutes and Glycine/HCl desorbing the IgG fraction for 2 to 20 minutes.

4.22., separation was effected by the delivery of Tris/HCl for 0 to 20 minutes, followed by the desorption of the IgG component using the glycine/HCl buffer for the subsequent 20 to 35 minutes. Reducing the loading time of the Tris/HCl buffer gradually from 20 to 15 (Figure 4.23.) to 12 (Figure 4.24) to 2 minutes in a series of experimental runs, greatly reduced analysis time. In both these examples, the glycine/HCl buffer eluted the IgG component for a further 20 minutes. Figure 4.25. illustrates this shorter run time and the resulting improved chromatogram as well as an improved injection technique. (The sample was filtered through the Millex GV filter first, as opposed to filtering through the Millex GV filter while the sample was being injected onto the Memsep cartridge.) The baseline perturbation at 19 to 20 minutes is due to column re-equilibration and refractive index effects as the new solvent passed through the analytical flow cell.

Gradients and flow rates can thus be optimized as follows:
Tris/HCl buffer - 1.5 ml/min - 0 to 2 minutes
glycine/HCl buffer - 1.5 ml/min - 2 to 20 minutes

4.2.3.8.3 Choice of Rheodyne Injector Loop Volume

The Waters Delta Prep 4000 system was supplied with a series of rheodyne loops aimed at delivering a fixed volume of sample to a column or cartridge. In this manner, inaccuracies due to manual volume selection by a syringe are avoided. The HPLC Rheodyne 7010 injector was supplied with two 2.5 ml loops as well as one 100 μl loop. These three loops could be configured in any combination to change the injection volume.

When using diluted samples for analysis, a single 2.5 ml loop was too large for the volumes of serum available. Hence, this loop was replaced with a 500 μl loop and the majority of the developmental work regarding antibody

purification was performed using this loop size. However, the chromatograms yielding the best resolved serum protein and IgG peaks are obtained from the use of a 100 μl loop (Figures 4.26., 4.27. and 4.28.). Whichever loop was used, ten times the volume of the loop was drawn into the syringe to ensure the loop was completely filled prior to the sample moving to the Memsep cartridge. Excess sample went to waste and was re-injected if required. Accuracy of injection was calculated to be 0.5% by multiple injection of a standard sample.

Millipore suggested that while a run was in operation (and the rheodyne injector in the "inject" position), the loop be rinsed with a volume of mobile phase (Tris/HCl, in this work). The mobile phase went directly to waste and thus did not affect the chromatography, while the loop was cleared of any adhering sample which might affect subsequent analysis.

4.2.3.8.4 Optimization of Integrator Parameters

With the fundamental chromatographic settings of the HPLC optimized as discussed in Sections 4.2.3.8.1 to 4.2.3.8.3, this section discusses ways in which the digital signal from the detector can be altered to display differently. This is brought about by altering some of the pre-set parameters of the Waters 745 data module. A chart speed of 0.5 cm/min was set and maintained throughout this work. A peak threshold (PT) of 500 was set initially. This value indicated to the integrator function that any integrated peak areas greater than 500 must be responded to and represented on the chromatogram. Setting the PT value to 3000 meant that a large portion of the noise on the IgG fraction was eliminated. This is clearly illustrated in Figure 4.29. where a significant reduction in signal-to-noise ratio can be seen in comparison with Figure 4.24. where the PT value was set at 500.

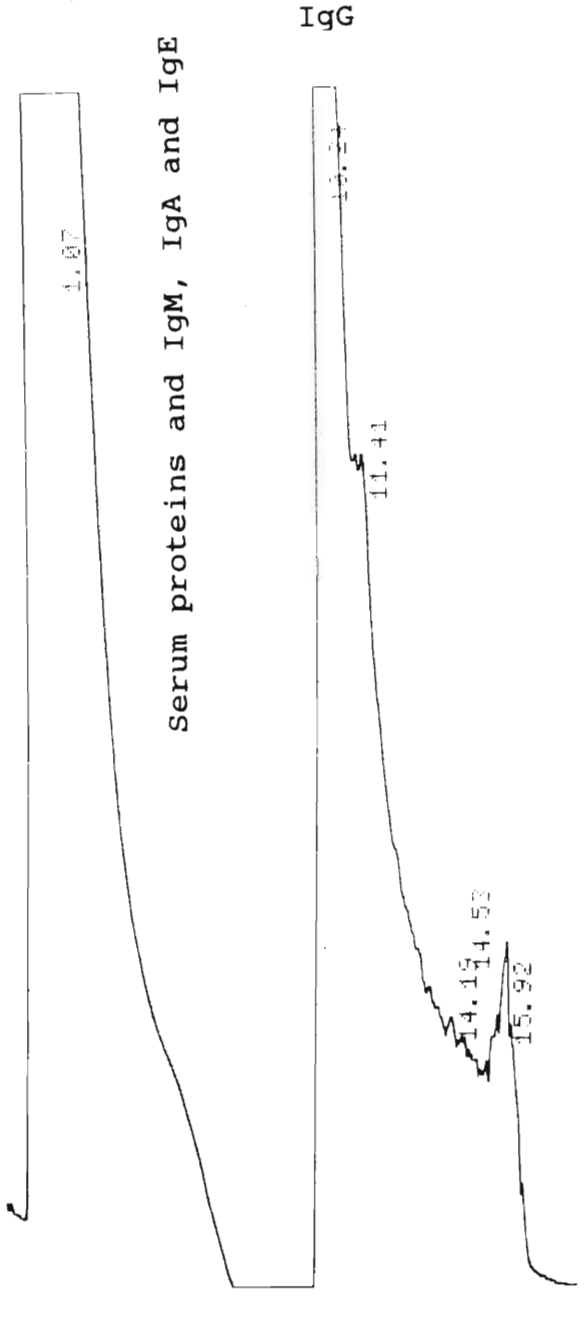


Figure 4.26. HPLC chromatogram illustrating optimum separation of the IgG fraction from the serum proteins and other immunoglobulins using a 100 µl loop volume (Attenuation set at 32).

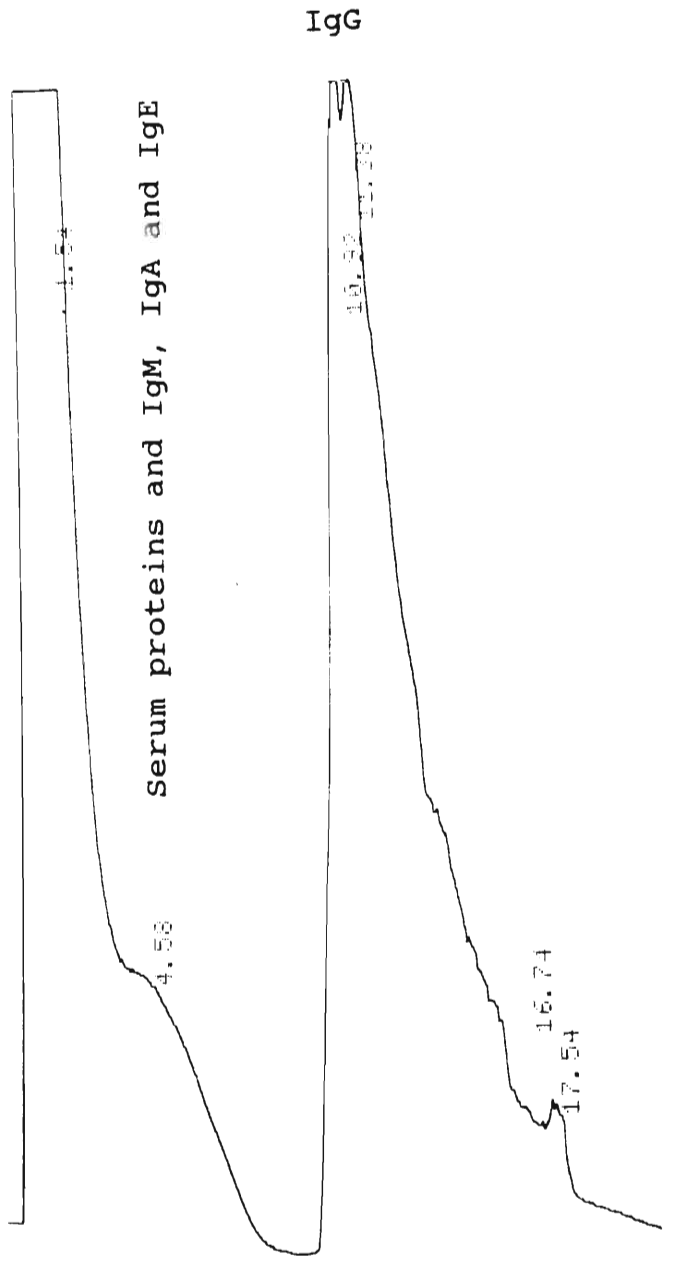


Figure 4.27. HPLC chromatogram illustrating optimum separation of the IgG fraction from the serum proteins and other immunoglobulins using a 100 µl loop volume (Attentuation set at 64).

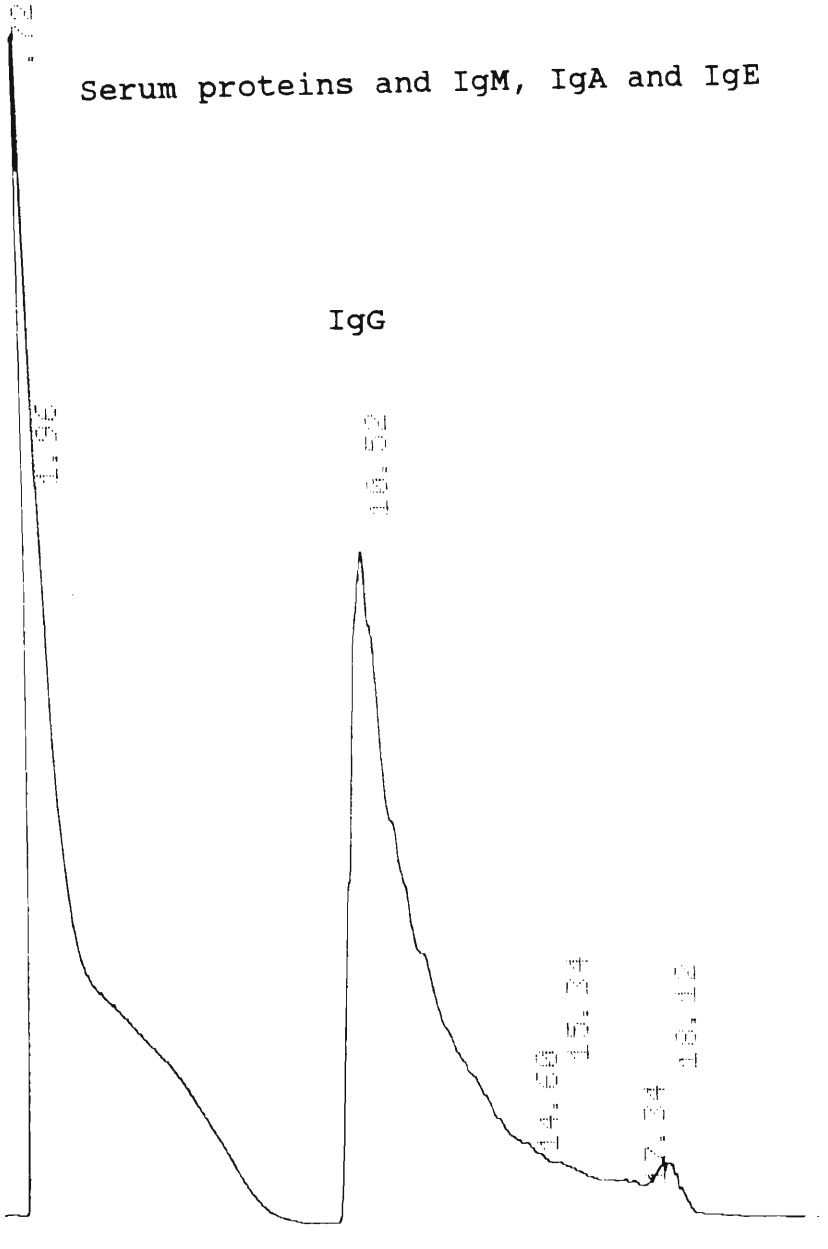


Figure 4.28. HPLC chromatogram illustrating optimum separation of the IgG fraction from the serum proteins and other immunoglobulins using a 100 µl loop volume (Attenuation set at 128).

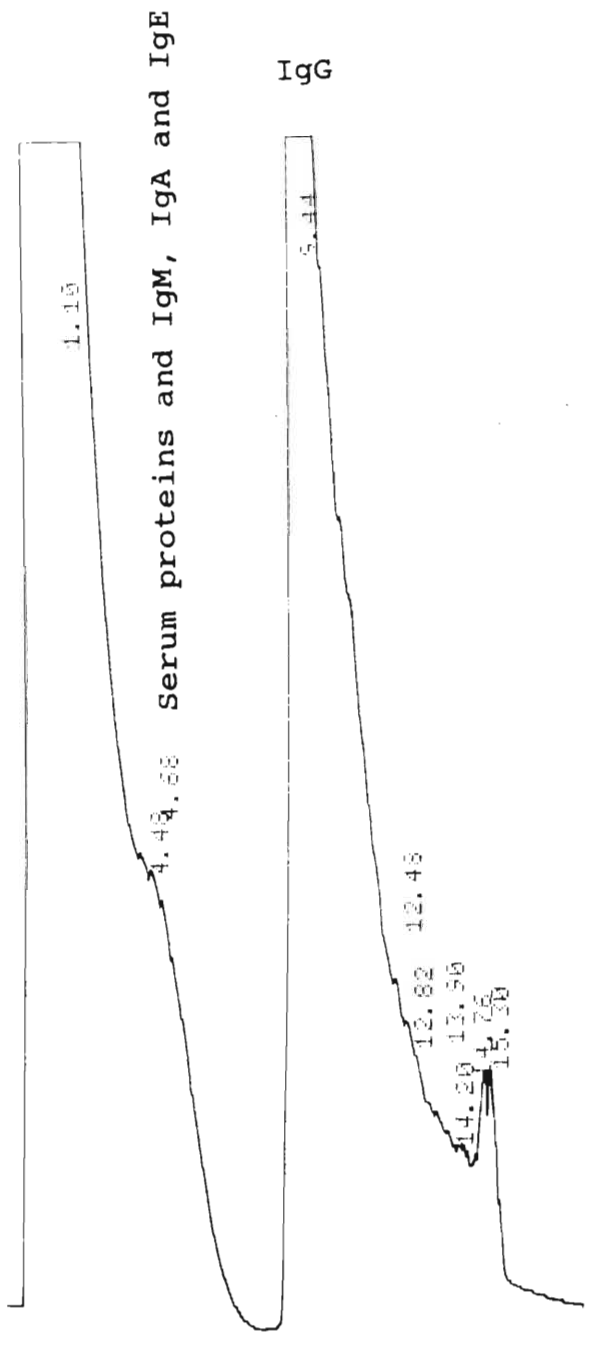


Figure 4.29. HPLC chromatogram illustrating optimum separation of the IgG fraction from the serum proteins and other immunoglobulins with loop volume = 100 µl, attenuation = 32 and peak threshold = 3000.

However, no one chromatographic parameter will adequately effect resolution of a chromatogram. In this regard, the peak width (PW) and attenuation (Atten) which are pre-programmed into the data module radically affect the manner in which the chromatogram is displayed. Peak width values alter the breadth of the serum protein and IgG peak. The default PW setting of 6 was used in the developmental stages of this work, but when resolution of the serum protein from the IgG fraction was important, PW values of 30 and 40 were experimented with, and a value of PW = 30 was finally settled on. Figure 4.30. illustrates a PW = 40, all other parameters except flow rate of 2.0 ml/min being held constant.

The attenuation setting affected the full scale deflection of the pen across the width of the chromatographic paper. Values of 1 (default), 2, 4, 8, 16, etc. were programmed into the data module and the attenuation value altered according to the individual serum sample. In this work, attenuation values less than 16 caused extensive baseline noise and were not used. In the developmental stages of the antibody purification, attenuations of 32 and 64 were used, with a setting of 128 allowing all the serum protein signal to be displayed. Figures 4.26., 4.27. and 4.28. illustrate attenuation settings of 32, 64 and 128, respectively for the same serum sample with the Tris/HCl buffer loading for 2 minutes and the glycine/HCl desorbing the IgG fraction for a subsequent 18 minutes. In these figures PT = 3000 and PW = 30 were used, as well as a loop volume of 100 μl.

It must be stressed, however, that the parameters used in this work are specific for the serum sample under investigation. They do give an indication, though, of the nature of the parameters which are applicable to purification work of this type, and the way in which they interact, constructively or destructively, to bring about chromatographic resolution.

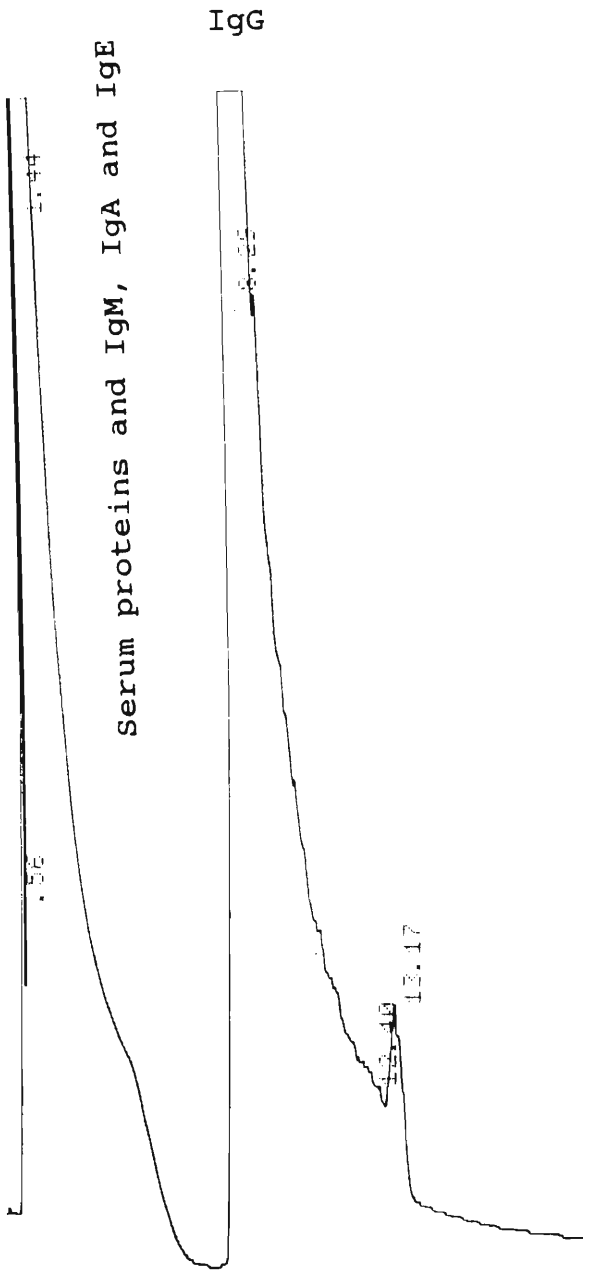


Figure 4.30. HPLC chromatogram illustrating optimum separation of the IgG fraction from the serum proteins and other immunoglobulins with loop volume = 100 µl, attenuation = 32, peak threshold = 3000 and peak width = 40.

4.2.3.9 Collection of IgG Fractions

Despite the fact that only two peaks are evident on the chromatograms, a series of runs with commercial rabbit IgG were performed to confirm the retention time and presence of the IgG fraction. As outlined in Section 4.2.3.1, rabbit IgG was weighed out and dissolved in PBS buffer. Confirmation was performed by injecting the commercial IgG fraction in PBS onto the Memsep cartridge and noting the retention time. This IgG sample was collected and re-injected under identical operating conditions and the retention time noted. A serum sample was also spiked with commercial IgG and the increase in absorbance of the IgG peak at the relevant retention time noted.

However, fraction collection was only performed when the waste line, originally 1 metre long, was replaced with a 3 to 4 cm length of 40" tubing connected to the outlet of the analytical detection cell. Thus, as the increase in absorption at 280 nm was registered on the detector, the sample was simultaneously collected until the absorbance of the detector dropped back to zero.

For a 0.96 mg/ml rabbit IgG sample injected using a 100 μl loop and attenuation of 32, a serum component at 1.12 minutes is evident in Figure 4.31. This 7% contamination can be attributed to the serum protein present in the prepared sample, or contamination of the rheodyne loop itself. The sample was collected between 14.2 minutes and 25.2 minutes. At a 1.5 ml/min flow rate, 17.5 ml of sample was collected, effectively diluting the IgG to 0.00547 mg/ml. This concentration of IgG was not detectable on the HPLC even at low attenuation values of the data module. Hence, a more concentrated IgG solution of 2.42.mg/ml was prepared, injected onto the Memsep and the fraction between 11.90 minutes and 21.90 minutes was collected. The concentration of the IgG was effectively reduced to 0.016

Figure 4.31. HPLC chromatogram illustrating a 0.96 mg/ml commercial rabbit IgG sample passed through the Memsep cartridge under optimum operating conditions.

mg/ml. This sample was re-injected at a series of attenuation settings (16, 32 and 64). Because the sample was diluted, spreading of the peak was evident on the chromatogram, but the retention time remained constant in all cases that were tested (Figure 4.32.). This suggested that the second peak was indeed IgG. This was corroborated by spiking a serum sample with commercial IgG and noting the increase in peak area at the same retention time. An additional confirmatory test was attempted. The UV absorbance of the diluted IgG against a Tris/HCl blank was measured. However, because of the extremely dilute IgG sample, no UV absorbance was noted.

The final section provides some comments on the interpretation of the chromatogram.

4.2.3.10 Interpretation of HPLC Chromatogram

Using anion-exchange chromatography and carefully controlled gradients, Gemski et al.[157] identified transferrin and albumin as those serum proteins present in a sample of tissue culture fluid. It is likely that these same contaminants are present in mammalian serum samples.

Seeing that a single peak (or shoulder and a peak) is evident in the chromatograms in this work, and because the Tris/HCl buffer was used without a concentration gradient, it is likely that the serum components plus any IgE, IgA, or IgM immunoglobulins co-elute. This postulate could have been tested by spiking a serum sample with the relevant serum protein or immunoglobulin. However, these biochemicals are prohibitively expensive and confirmation of their presence served no purpose in this work. However, Ey, Prowse and Jenkin[158] do confirm that IgM, IgA and IgE antibodies do not show significant binding to a Protein A column and can be collected with other serum proteins in the effluent.

Figure 4.32. HPLC chromatogram confirming the retention time of rabbit IgG at 17 minutes.

Chapter 5 now discusses the immunoassay protocol that was chosen in this work to detect and quantitate thymine dimers in UV-irradiated DNA at concentrations currently undetectable by HPLC.

CHAPTER 5 IMMUNOASSAY TECHNIQUES

5.1 INTRODUCTION

This chapter describes the choice and development of an immunoblotting protocol for the detection and quantitation of thymine dimer in UV-irradiated DNA. This section (5.1) provides a brief introduction to the immunoblotting protocol chosen in this work for the detection of UV-DNA antigen and antibodies generated against it. First the discussion will focus on a description of the technique, then a review of thymine dimer detection techniques using immunoblotting will be given.

5.1.1 DESCRIPTION OF THE IMMUNOBLOTTING DETECTION PROTOCOL

Immunoblotting is a general term used to describe a powerful molecular technique for the identification of specific antigens. Use is made of affinity interactions between an antigen and an antibody generated in response to the antigen. These interactions take place when the antigen, which has been immobilized on a membrane, is exposed to the antibody. Measurement of the subsequent binding of the antibody to the antigen allows quantitation of the membrane bound antigen to be made. The aim of the immunoblotting protocol developed in this work was the quantitation of thymine dimer lesions in ultraviolet-irradiated DNA. After being transferred to the membrane by "dot blotting", the UV-irradiated DNA was immobilized there. Antibodies generated against the UV-DNA antigen were then added. The antibodies bound to the antigen located on the membrane surface and the degree of binding (and hence antigen concentration) were quantified. A more detailed description follows.

Immunoblotting was first described by E.M. Southern [83] in 1975 for the detection of particular sequences of single stranded DNA in a mixture of DNA fragments and has become known as "Southern Blotting". When used in molecular biology applications, Southern Blotting involves the following protocol. Restriction enzymes are used to recognise a specific nucleotide sequence within the DNA and then to cleave both strands of the DNA at the location of that sequence. The restriction-enzyme digested DNA is then fractionated by electrophoresis within an agarose gel medium. The next step is the transferral of the separated fragments to a suitable support membrane by means of a "blotting" procedure. Once transferred, the DNA must be fixed on the suppport medium. In the original Southern Blotting protocol, this is accomplished by baking the membrane for several hours in a warm oven, after which a protocol may be followed to allow for the addition of antibody specific for the sequence of interest, and for the assessment of the degree of antibody binding. This subsequently allows the concentration of the DNA sequence to be measured.

An obvious extension of this idea has been the development of a technique for the identification of specific RNA sequences within a population of RNA molecules. This technique has been named "Northern Blotting". Similarly, a technique for the identification of proteins and other biomolecules (e.g. DNA components) has become known as "Western Blotting".

An immunoassay can be described as a technique or protocol allowing for the identification or quantitation of antibodies. For the development of an immunoassay to detect thymine dimers in UV-irradiated DNA, the specific immunoassay technique called "Western Blotting" or simply "Immunoblotting" was adapted to allow the spotting of the antigen directly onto a membrane (using a micropipette)

rather than to transfer it electrophoretically from a gel. Once the antigen was spotted on the membrane, all remaining sites on the membrane (i.e. those which have not been covered with the antigen) are blocked using a suitable reagent (e.g. 2% dried milk powder). The membrane is then incubated or immersed in the crude serum containing the antibodies and after washing with a detergent (e.g. 0.1% Tween-20), the membrane (which now holds bound antigen/antibody complexes) is incubated with a secondary antibody conjugated to an enzyme system. The secondary antibody/enzyme complex binds to the antigen/antibody complex.

As will be discussed later in this work (Section 5.2.2), the enzyme is chosen to catalyse a light reaction. The intensity of the light emitted is then used to quantitate the antibody and antigen systems used in this work. The immobilization of all components on the membrane surface is illustrated in Figure 5.1.

For the purpose of this work, the manufacturer's protocol as supplied by Amersham, was used for the development of an immunoblotting technique (Section 5.2.1.7). The procedure used by Amersham is a very general one, and had to be tailored to the specific requirements of quantitating thymine dimers in UV-irradiated DNA.

The following section is a review of immunoblotting techniques which have been used by previous workers to develop an immunoassay method for thymine dimer quantitation.

5.1.2 REVIEW OF THYMINE DIMER DETECTION TECHNIQUES USING IMMUNOBLOTTING

This section seeks to survey previous work which quantified pyrimidine dimers by means of immunoassay. As was outlined

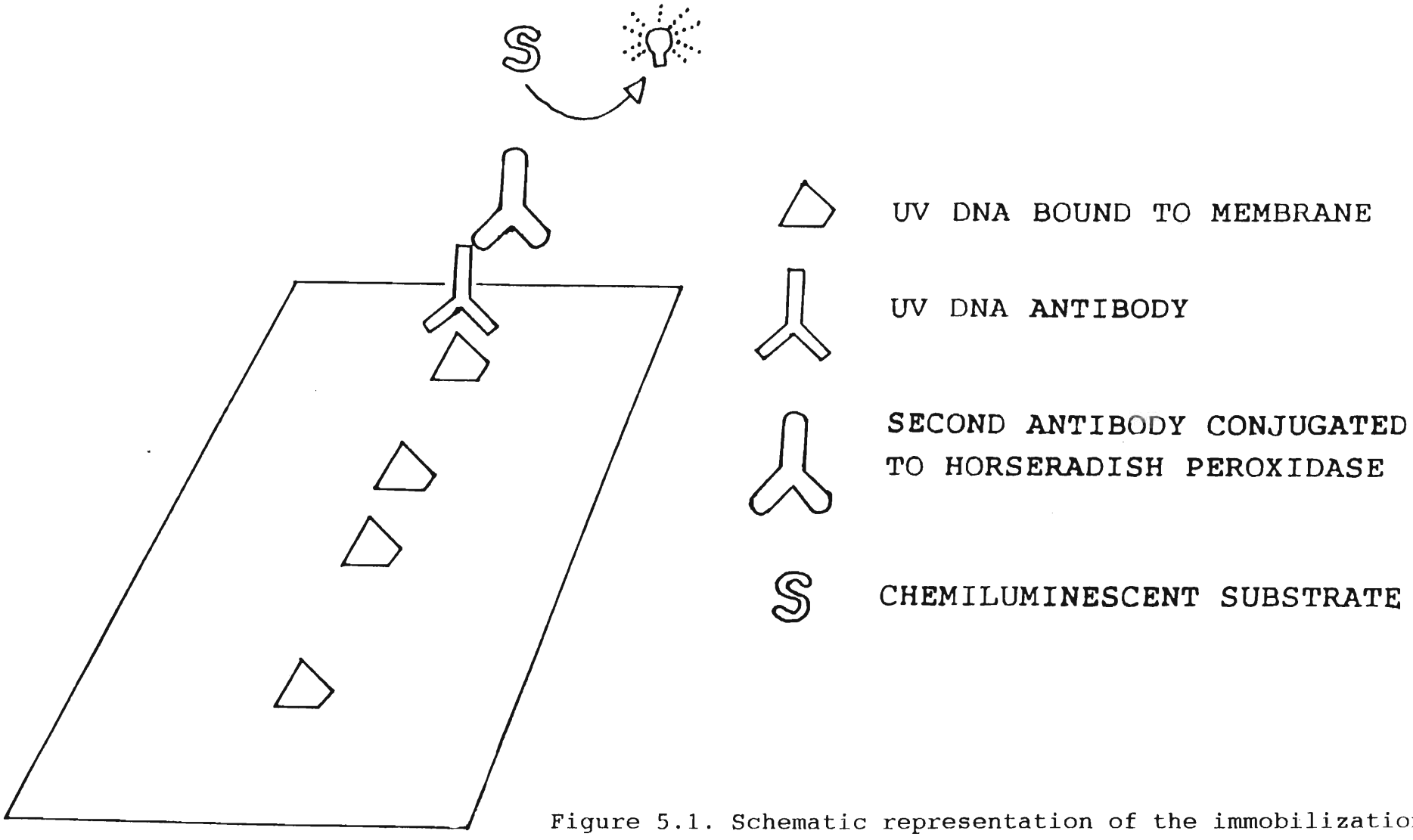


Figure 5.1. Schematic representation of the immobilization of antigen, antibody and secondary antibody on the membrane surface, together with the chemiluminescent substrate.

above, immunoassay is the general term for any technique which quantitates antibody by the detection techniques of radioimmunoassay, fluorescence detection (immunofluorescence) or ELISA. Because an immuno technique can be used for detection and quantitation, detection only (as in this work) or quantitation only, some of the discussion which is to follow serves to strengthen and extend the comments and observations made in Section 1.2.

In this Section (5.1.2), the discussion will first centre around a review of the immuno techniques that were used to quantitate thymine dimers generated in UV-irradiated DNA systems. Then the discussion will extend to previous work performed to quantitate thymine dimer specifically by means of immunoblotting; that is, blotting of the UV-DNA antigen onto a membrane surface followed by incubation of the blot with antibody. Subsequent detection and quantitation using one of several techniques (scintillation counting or fluorescence tagging) is then performed.

The development of immunoassays for the detection of modified nucleic acid components began more than 20 years ago with the demonstration that these compounds are antigenic under certain conditions[66,67,68,78,132]. Since that time, a wide variety of antibodies have been produced that are specific for DNA components modified by many different carcinogens, both chemical and physical in nature[70].

Several immunoassay methodologies have been used for the detection of modified nucleic acid components including pyrimidine dimers generated in irradiated cellular DNA. For instance, Tan and Stoughton[159] demonstrated the existence of UV-induced lesions in DNA in cell nuclei of irradiated tissue sections (and human skin) by using light microscopy to observe antibodies labelled with a fluorescent tag. These immunofluorescent techniques are not sufficiently

sensitive to easily reveal lesions induced by low fluences of radiation (less than 10 J/m^2) and, in addition, require some fairly sophisticated fluorescence measurement instrumentation. As an alternative, radiolabelling of the antibodies (^3H or ^{125}I) can be used (radioimmunoassay). Seaman et al.[74] sucesssfully used this technique as a means of detection of thymine dimer removal in bacterial and mammalian cells. Cornelis et al.[72] used ^{125}I labelled antibody directed against UV-irradiated DNA to measure pyrimidine dimers *in situ*.

One technique which does not rely on radiolabelled DNA or fluorescence tagging of the antigen is the Enzyme Linked Immunosorbent Assay (ELISA). This technique has been used for detection of pyrimidine dimers in DNA and is similar to immunoblotting in that the antigen, antibody and secondary antibody conjugated to an enzyme are immobilized on a support medium. The support medium is a 96 well plastic ELISA plate, and it is the well which provides the surface of attachment for the immune complex. When a substrate (e.g. 5-amino-2-hydroxybenzoic acid) is added to the complex, the enzyme catalyses the conversion of the substrate to a chromogenic reaction product. Quantitating the colour intensity produced during the course of the reaction allows the bound antibody, and hence the bound antigen, to be quantitated.

A microELISA technique was successfully implemented by Leipold and co-workers[82] for the quantitation of photolesions using UV-irradiated DNA coupled to the wells of an ELISA plate. Lesions induced by UV irradiation doses as low as 2.5 J/m^2 were detected using 8 μg of sample.

Work has been performed in this laboratory on the quantitation of thymine dimers in aqueous thymine and in *in vitro* DNA systems. The development of an immunoassay technique in these laboratories is designed to extend this

work to quantitation of thymine dimers in cellular systems of UV-irradiated DNA. Here, the cells are to be irradiated, the DNA extracted and the thymine dimers quantitated using an immunoassay. (The HPLC techniques used for the *in vitro* studies are too insensitive for the quantities of DNA involved.) Because of the hazards of using radioactive material in the laboratory, and because of the longer shelf-life of non-isotopic labels, the radioimmunoassay technique for thymine dimer quantitation was not chosen. Fluorescence tagging is not strictly a quantitative technique[160], and would not yield the levels of sensitivity required in this work.

However, immunoblotting, does offer an appropriate method. Recently, an immunoblotting technique was described by Nehls et al.[84] for the quantitation of carcinogen-modified nucleosides in DNA. Femtomole to picomole amounts of these nucleosides were detected using a radioimmunoassay method of quantitation. Nehls et al.[84] propose that this method yields significantly lower detection limits than the radioimmunoassay (used for detection) which was also performed on these modified nucleosides. Comparable sensitivity to immunoblotting can be achieved using an ELISA, but reproducibility is often a problem, partly due to variations in absorption of the antigen onto the wells of the plastic ELISA plate. The membrane system used by Nehls et al.[84] appeared to provide a reliable support for immobilization of DNA.

Plaza and co-workers[161] in 1991 used an immunoblotting protocol to demonstrate repair kinetics of UV-DNA damage using an antigen specific for pyrimidine dimers as well as the other photolesion, the 6-4 photoproduct. Wani et al.[162] used an immunoblotting technique for detection of pyrimidine dimers in non-labelled skin fibroblasts and calf thymus DNA irradiated at sub-lethal (0.5 J/m^2) doses of UV. The observation that this technique could detect thymine

dimers generated at sub-lethal UV fluences, added weight in support of choosing immunoblotting as the technique for quantitation of thymine dimers in UV-irradiated DNA.

The following section describes the development of a "tailored" protocol for this work, from the choice of reagents and equipment to the development of a technique which delivered reproducible results. First, the choice of immobilizing membrane will be discussed, followed by the choice of diluent buffer. Thereafter will follow a discussion concerning the choice of detergent wash solution that was chosen for use, as well as the choice of blocking reagent. The choice of secondary antibody will also be discussed, together with the equipment that was chosen for use in this work. Once the choice of reagents and equipment have been discussed, this chapter will extend to the development of the immunoassay protocol for thymine dimer detection in UV-irradiated DNA.

5.2 EXPERIMENTAL TECHNIQUES

5.2.1 DEVELOPMENT OF A PROTOCOL FOR ANTIBODY IMMOBILIZATION

This section seeks to discuss the choice of reagents (Section 5.2.1.1 to Section 5.2.1.5) and equipment (Section 5.2.1.6) used in this work. Thereafter the development of a protocol for antibody detection will be discussed in terms of the chosen reagents and equipment.

5.2.1.1 Choice of Membrane

Immunoblotting protocols require a support medium upon which all antibody/antigen interactions take place. A membrane system is most commonly adopted, although absorption onto polystyrene can also be used. The advantages of using a membrane system include accessibility

of the antigen to the appropriate antibody, shorter reaction times for reagent immobilization and economy of reagents. These advantages led to the use of a membrane system being favoured as the immunoblotting matrix. Numerous membranes are commercially available, with nitrocellulose being the most widely used material[163]. Originally nitrocellulose membranes were used for the microfiltration of bacteria but they are now also used for immunoblotting. In this latter application the antigen and antibody are adsorbed throughout the matrix predominantly by chemical interaction. Binding by chemical interaction is important in enhancing the attachment of the immune complex to the membrane and facilitating its detection on the luminescence film once the chemiluminescence detection protocol has been followed (Section 5.2.2.3). During the development of the immunoblotting technique in the 1980's, many of the antigens which were used for blotting purposes were proteins. It was found that proteins, and especially those of low molecular weight, bound to nitrocellulose with low affinities and were lost during subsequent processing[163]. This had the obvious disadvantage of not allowing the immune complex to be visualized on the luminescence film during detection.

Because of this problem of low affinity binding of the proteinaceous antigen to the nitrocellulose membrane, alternative matrices for protein blotting were developed. Diazobenzyloxymethyl-modified cellulose paper has been used[164], but had the problem of loss of antigen sample when the antigen is transferred from the agarose gel to a modified cellulose support medium. To overcome this problem, other membranes have been developed and include diazophenylether paper[165] as well as nylon based membranes. These latter two membranes contain positively charged diazonium or tertiary amino groups introduced during the manufacturing process. This ensures less antigen loss because of an enhanced binding capacity of the antigen

to the membrane. This, in turn, increases the sensitivity capacity of the immunoblotting technique because of electrostatic interactions between the membrane and polyanions in the antigen. This observation is supported by Gershoni and Palade[163] who determined the binding capacity of the commercially available charge modified membrane, Zetabind. Their work indicated that this membrane has a binding capacity for proteins of 480 $\mu g/cm^2$, in comparison with unmodified membranes which have binding capacities of approximately 80 $\mu g/cm^2$.

In this work, in order to ensure all thymine dimer containing DNA antigen was bound to the membrane, a charge modified membrane was chosen for immunoassay development. The membrane chosen (polyvinylidene difluoride, PVDF) has specific applications to binding nucleic acids or nucleic acid components and is commercially available as Immobilon-N, a high strength and durable charge modified membrane. The membrane exhibits high DNA binding which may be attributed to the combination of the charge modified surface and the chemical nature of the PVDF. Ionic interactions (and hence binding) between the membrane and the polyanions of the phosphate backbone of DNA are enhanced by means of the charge modified membrane surface, while the fluorocarbon backbone of the PVDF membrane contributes to hydrophobic interactions between the membrane and the nucleotide bases. These characteristics also help retain blocking agents added at a later stage of the protocol. This last-mentioned aspect ensures that binding of a non-specific macromolecule to the membrane itself does not occur, thereby facilitating detection of the immune complex against a clear background. Other advantageous features of the PVDF membrane include its hydrophobic nature, implying that it is compatible with acids and is highly resistant to organic solvents. This allows vigorous washing routines which also remove non-specific protein molecules that may bind to the membrane

itself.

According to Millipore, Immobilon-N was also compared to three other commmercially available membranes in a Southern Blotting test protocol. A nitrocellulose membrane (BA-85), a nylon membrane (Hybond), a charge-modified nylon membrane (Genescreen) and Immobilon-N were subject to blotting of three dilutions of Lambda DNA. The Immobilon-N charge modified PVDF membrane showed the highest sensitivity of all membranes for detecting low concentrations of DNA (0.01 μg DNA). At the highest loading of 0.20 μg DNA, the Immobilon-N showed a more intense signal when detected by radioimmunoassay. Thus the Immobilon-N was shown to be the most effective of the membranes tested for nucleic acid binding.

5.2.1.2 Choice of Diluent

An immunoblotting protocol requires extensive washing of the membranes at each stage of the protocol, in order to remove extraneous solution that might enhance, instead of reduce, non-specific binding of biomolecules to the membrane. Usually a detergent that is dissolved in a diluent buffer is used to achieve this. The crude serum samples and secondary antibody are also diluted using this same buffer. As discussed in Section 2.8.2.5, all DNA samples were prepared in 0.15 M phosphate buffered saline, pH 7.4 (PBS) in order to reproduce physiological conditions. As a diluent in the immunoblotting protocol, the manufacturer (Amersham) suggested the use of tris buffered saline, pH 7.6 or phosphate buffered saline, pH 7.4. Because PBS was already being used as a buffer in the DNA work, it was decided to simplify solution preparation by using PBS for immunoblotting as well.

Solutions of PBS were prepared by weighing 8 g of NaCl, 0.12 g of KH_2PO_4, 0.20 g of KCl and 0.91 g of Na_2HPO_4.

Analytical grade reagents were used in all cases. This gave a 0.15 M PBS solution. The salts were dissolved in a small volume (100 ml) of Milli-Q water in a beaker and then made up to 1 litre in a volumetric flask. The pH of the buffer was measured using a Jenko portable pH meter calibrated against UniVAR pH 7.0 and pH 4.0 buffer solutions. The pH of the buffer solution was between 7.3 and 7.4 in all cases. The PBS was stored in the refrigerator and was vacuum filtered prior to use in order to remove any particulate matter.

5.2.1.3 Choice of Detergent Wash Solution

In order to wash the membranes, the manufacturer (Amersham) suggested the use of 3% Tween-20. (As discussed above, the Tween-20 was dissolved in PBS.) Fonong et al.[166] also used this solution when detecting autoantibodies in human serum. Leipold et al.[82] suggested the use of 0.05% Tween-20 in PBS, and Thomas et al.[167] used 0.1% Tween-20 in PBS. A 0.05% Tween-20 in PBS was also used by Mizuno et al.[168] in an ELISA protocol that established a monoclonal antibody which recognized cyclobutane-type thymine dimers in DNA. Another detergent which has been used on a regular basis for washing membranes is 0.1% Triton X-100 (a polyoxyethylene ether non-ionic detergent) in PBS[84].

The protocol as used by Leipold and co-workers[82] was used as a starting point in developing an immunoassay, and thus it was decided to adopt the use of Tween-20. Once a few minor problems concerning the use of Tween-20 had been identified (see below), this reagent was used throughout the developmental and data collection stages.

Analytical Grade Riedel-de-Haën Tween-20 (Polyoxyethylenesorbitan monolaurate, density 1.10 g/cm^3) was used. Initially a 3% solution in PBS (PBS-T) was prepared. This was used in a few initial runs, but the

concentration was excessively high (even though specified by the manufacturer) and resulted in the antigen/antibody complex being washed from the membrane before detection. Amersham did, however, note that high concentrations of Tween-20 may reduce the binding of antibodies, particularly low affinity antibodies. A literature search was undertaken and it was found that far lower concentrations than the 3% solution of Tween-20 were generally used[82,167]. Concentrations were usually in the 0.05% to 0.1% range. When a 0.1% PBS-T was used and an immunoblotting protocol performed, antigen/antibody complexes were not washed from the membrane and were sucessfully detected on the luminescence film (Section 5.2.2.3). Hence a 0.1% Tween-20 solution in PBS was used in all washing steps.

Tween-20 is a lumpy, yellow liquid which could not be weighed or transferred easily. It was decided to use a syringe or micropipette to deliver a reproducible volume of detergent to the PBS buffer. It was usual to prepare 1 litre of solution at a time since this volume was sufficient for two entire protocols. In order to prepare a 0.1% solution of Tween-20 in PBS (density of Tween-20 = 1.10 g/cm^3, it was necessary to deliver 0.909 ml of Tween-20 to 1 litre of filtered PBS. Initially a micropipette was used to deliver the 0.909 ml, but because the detergent was too viscous, it could not be drawn into the narrow pipette tip. Hence a 2.50 ml graduated plastic syringe was used to draw up 0.90 ml of Tween-20. By eliminating all air bubbles in the detergent when drawing up, this was an easy task to perform. This volume (0.90 ml) was then transferred to a volumetric flask which was then made up to 1 litre with filtered PBS.

Being viscous, the Tween-20 did not dissolve instantaneously in the PBS and in order to ensure complete solvation, the solution was sonicated for approximately 30 minutes with occasional shaking of the flask by hand.

The detergent was stored at room temperature and because only 0.90 ml was required to prepare 1 litre of solution, an aliquot (50 ml) of the reagent was transferred to a vial. It was important that a glass vial as opposed to a plastic vial was used for storage to prevent compounds from diffusing into the Tween-20 from the plastic and affecting its detergent functions.

5.2.1.4 Choice of Blocking Reagent

Several reagents have been used by researchers in order to block sites on the membrane not occupied by antibody/antigen interactions. Such a step is crucial in immunoblotting protocols as it serves to reduce non-specific protein binding (e.g. binding of secondary antibody directly to the membrane) and hence simplifies identification of the immune complex against a clear, as opposed to a darkened background on the luminescence film.

Blocking is most commonly achieved by incubating the blot in a high concentration of bovine serum albumin, horse serum albumin , fetal calf serum, haemoglobin, ovalbumin or gelatin. Hawkes et al.[169] suggests the use of 3% (w/v) bovine serum albumin and 1% (v/v) normal goat serum in tris buffered saline. More common is the use of a dried milk solution dissolved in the PBS-Tween wash solution. Such a reagent has been used by Nehls[84], Fonong[166] and Thomas[167] as well as being specified by the manufacturer of the immunoblotting reagents and equipment (Amersham).

The type of low fat milk powder is apparently not crucial and Farmer's Pride low fat milk powder was bought from a supermarket and stored in a dessicator. Initially a 5% solution was prepared by weighing out 25 g of milk powder. This was dissolved in 200 ml of PBS-T on a magnetic stirrer and then made up to 500 ml in a volumetric flask. The solution was then stored in a refrigerator until required.

After a few runs had been performed, it was decided to reduce the concentration to 2% dried milk in PBS-T. Seeing that similar background levels were visually observed on the luminescence film when compared to 5% dried milk blocking solution, it was decided to use this lower concentration in all subsequent protocols. The solution was stored as outlined above. When a working protocol was being used regularly, it was found that the dried milk dissolved in solution began to coagulate after a 3 to 4 week period. The solution thus had to be discarded and prepared fresh monthly.

It was usual for several membranes to be treated together when the protocol was being performed. Usually 6 to 9 membranes could be accommodated on the modified ELISA plate when blotting the membranes (Section 5.2.1.7.2), and in the plastic box when washing the membranes (Sections 5.2.1.7.4, 5.2.1.7.6 and 5.2.1.7.8). If 6 to 9 membranes were processed together during an immunoblotting protocol, it was found that 20 ml of 2% dried milk in PBS-T gave sufficiently low background results. This will, however, be discussed in more detail in Section 5.2.1.7.3.

5.2.1.5 Choice of Secondary Antibody

In order to detect the antigen/antibody complex immobilized on the membrane, a suitable visualization system had to be employed. It was decided to add a second rabbit antibody to the antigen/antibody complex on the membrane. Under incubating conditions this would bind to the immobilized complex. Binding between the two antibodies will take place because both are rabbit antibodies and hence have similar antigenic determinants and binding domains.

For immunoassay purposes this secondary antibody has the added feature of itself being conjugated to an enzyme (horseradish peroxidase), which in the presence of a

suitable substrate (luminol), will catalyse the conversion of luminol to the aminophthalate dianion and light of 425 nm will be emitted in the process. The emitted light intensity is proportional to the amount of horseradish peroxidase bound to the membrane which in turn is proportional to the amount of secondary antibody bound to primary antibody, and this is proportional to the amount of antigen bound directly to the membrane. Thus the emitted light intensity can be used to quantitate the antigen bound to the membrane. When a luminescence film is imposed on the membrane and luminol added to the complex, the light emitted impinges on the film. The film can then be developed and printed using standard photographic procedures to allow the intensity of the emitted light to be measured.

Such a technique of conjugating an antibody to an enzyme is used extensively in immunocytochemistry for the intracellular localization of antigens at the ultrastructural level[170]. The antibody/enzyme complex is incorporated into a cell or cellular system and binds to the selected antigen. When the substrate is added, light emission or an absorption producing chromogenic reaction, allows the localization of the antigen using electron microscopy.

Several antibody-enzyme systems are commercially available for the application required in this protocol. The horseradish peroxidase (HRP) system is widely used in immunoassay applications, especially immunoblotting and ELISA. In this application, suitable substrates for the enzyme must yield an insoluble coloured product on reaction. The intensity of the colour is then correlated to the concentration of analyte. The most popular of these substrates are diaminobenzidine, 4-chloro-1-naphthol and 3-amino-9-ethylcarbazole. A rabbit antibody (IgG)/HRP was chosen by Leipold et al.[82] for the quantitation of

photolesions in UV-DNA using an ELISA technique. It was also specified by Wani et al.[162] for the characterization of DNA damage induced by UV fluences as low as 2.5 J/m^2 in an ELISA application. HRP conjugated antibodies were also used for immunoblotting detection of antibodies developed against synaptosomal plasma membranes from rat brain[169].

Horseradish peroxidase is a low molecular weight (40 000 d) glycoprotein enzyme which also has applications as a marker in immunohistochemistry when coupled to immunoglobulin G[171]. Because of its small size it has superior penetration properties of cells and cellular systems when compared to other histochemical markers such as ferritin and fluorescein[170].

The antibody-enzyme system was supplied by Amersham as "Anti-rabbit Ig, peroxidase-linked species-specific whole antibody (from donkey)". The antibody was prepared by immunizing donkeys with purified immunoglobulin fractions from normal rabbit serum. This served to produce high affinity rabbit antibodies. However, to remove cross reacting antibodies towards human, rat and mouse immunoglobulins, the pooled serum was adsorbed using an affinity column packed with cross reacting human, rat and mouse antigens. Finally, to select for specific binding to rabbit immunoglobulins, the antibodies were purified using a specific affinity column containing rabbit serum and the required antibodies were eluted from the column using selected mild conditions which minimized aggregation and preserved immunologic activity of the antibodies. Mild conditions are important because immunologic functions may well be lost under harsh acid/base denaturing conditions.

Horseradish peroxidase (type VI from Sigma Chemical Company) was bound (by Amersham) to the purified immunoglobulin molecules using an adaptation of the periodate oxidation technique as proposed by Nakane and

Kawaoi[170]. Briefly, this involved the oxidation of HRP with sodium periodate in order to form aldehyde groups on the glycoprotein moeity of the enzyme. Addition of fluorodinitrobenzene blocked free α- and ϵ-amino groups and hydroxide groups on the enzyme. This prevented self-coupling and allowed the HRP-aldehyde to form Schiff bases with any protein (e.g. rabbit immunoglobulin) having α- or ϵ-amino groups. Such a coupling reaction has been found to be almost 100% efficient, since the conditions had been selected to minimize loss of enzymic activity of the HRP and immunologic activity of the antibody.

The antibody-enzyme system was supplied by Amersham in phosphate buffered saline, pH 7.5 containing bovine serum albumin (1% w/v) and an anti-microbial agent because the HRP-antibody system is subject to bacterial contamination if not used under sterile conditions. The product was stored in a refrigerator and according to Amersham was stable for 12 months under these conditions.

In Section 5.2.1.7.7 the working dilutions and incubation times chosen for the secondary antibody and for binding of the secondary antibody to the membrane bound antigen/antibody will be discussed in detail. In the following section, the choice of equipment used in the immunoassay will be discussed.

5.2.1.6 Choice of Equipment

As mentioned in Section 5.2.1.1, dot-blotting of the antigen onto the Immobilon-N membrane was performed manually using a micropipette set to deliver a specific volume of antigen to the membrane surface. However, all subsequent processing stages (interaction of the antigen with crude serum or secondary antibody on the membrane surface, as well as washing of the membranes) occurred by means of incubation of the membrane with the relevant

reagent.

One of the problems in immunodetection of blots is achieving an even coverage of the membranes with the various incubating solutions. The use of excessive volumes of expensive antibodies and detection solutions is also avoided.

Methods which have been most commonly used for processing (i.e. incubation and washing) of membranes include trays, plastic bags and boxes. The membrane remains flat within the box or tray and large volumes of reagents are used to totally cover the membrane. Reagent volumes can be reduced if plastic bags containing only a thin film of liquid are used. This method was adopted by Plaza et al.[161], but led to patchy results because of erratic reagent flow over the membranes themselves. Processing using plastic bags also has problems of leakage and contamination if radioactive reagents are used[167]. Other workers[172] used plastic tubes to process thin strips of membrane by rolling the tubes in detection reagent. The technique of using tubes has led to the development by Thomas[167] of a rolling drum system. Here, minimal volumes of reagents are used and all washing steps are carried out without handling the membranes themselves.

This seemed to be the best technique available and a shaking waterbath with a thermostat control was adapted to utilize its shaking motion to provide the "rolling motion" as used by Thomas. Such a technique was been succesfully implemented by Wani et al.[162] for the quantitation of pyrimidine dimers generated using sub-lethal doses of UV-irradiation on human cells.

In order to avoid the use of plastic bags or trays, all incubation procedures were carried out in 12 ml Polytop (number 3) glass vials (shown in Figure 5.2.). It was

Figure 5.2. Glass vial in which incubation of antibody, and later secondary antibody, with the Immobilon-N membrane was performed.

important to ensure that the lid of each vial sealed well, to prevent loss of incubating solution. Several vials (containing a single membrane each) were then placed in a plastic lunchbox. The lunchbox was then sealed and placed on the shaking waterbath for the required period of incubation.

Since this type of processing is dynamic, the membrane moves constantly throughout the reagent solution. This ensures that no localised depletion of reagent occurs, making the incubation very thorough and efficient. This results directly in increased binding of the immune complex to the membrane and hence ensures maximum light emission during detection. There is also no risk of uneven detection, and scratching or tearing of the membrane cannot take place during immunodetection.

This rolling drum technique was used successfully in the immunoassay protocol which is to be described in the following section.

5.2.1.7 Development and Use of a Working Protocol for Thymine Dimer Detection in UV-Irradiated DNA

The previous section described the choice of reagents and equipment used in the development of an immunoassay protocol for the identification of thymine dimers in UV-irradiated DNA. It also gave an indication of the reagent concentrations used in preparing solutions. This section now describes the specific nature of the protocol used in detecting and quantitating thymine dimers in UV-irradiated DNA.

5.2.1.7.1 Membrane Cutting and Wetting

As mentioned in Section 5.2.1.1, an Immobilon-N transfer membrane was chosen for the immunoblotting protocol because

its charge modified surface preferentially binds nucleic acids or nucleic acid components. It was supplied as 18 x 24 cm^2 sheets which were cut to the required size. This was best done using a ruler and pencil to mark 4 x 1 cm^2 sheets. These were cut using scissors and subsequently handled using a pair of fine tweezers. Such a precaution was imperative to prevent fingerprints obscuring any antigen/antibody blots on the luminescence film. After cutting, each membrane was labelled with a number or letter in the top left hand corner using a pencil. This served to identify each membrane during the processing and analysis stages.

Usually 9 membranes were transferred to a plastic box (10 x 8 x 6 cm^3) and pre-wetted. For this approximately 20 ml of 100% methanol was added to the box using a 20 ml beaker, and the membranes immersed for a maximum of 5 seconds. Pre-wetting could also have been achieved by immersing the membranes in 70% ethanol or 50% isopropanol. The membranes wet immediately in 100% methanol and changed colour from an opaque white to a uniform translucent grey. The methanol was decanted, and approximately 20 ml of Milli-Q water was added to the membranes in the plastic box using a 20 ml beaker. Immersion in water for 1 to 2 minutes served to flush away excess alcohol and it was important to submerge the membranes completely (they had a tendency to float) in order to effect complete alcohol to water exchange. The Milli-Q water was decanted from the box and approximately 20 ml of transfer buffer (PBS) was added using a 20 ml beaker. The membranes were allowed to equilibrate for 15 to 30 minutes in the PBS solution prior to blotting. If the membranes dried out at any stage of the immunoblotting protocol, it was important for the wetting protocol to be followed through again from the beginning.

5.2.1.7.2 Blotting of the UV-DNA Antigen

As mentioned in Section 5.1.1, Western Blotting generally refers to the electrophoretic separation of proteins or nucleic acid components on agarose gels, and the subsequent transfer of the component to an immobilizing matrix. In this work, the UV-DNA was transferred manually from a sample vial to the membrane surface using a micropipette. This is known as immunoblotting.

In this work, the membranes themselves could not be transferred from the PBS solution to an open benchtop prior to the blotting of the antigen because once blotted, the membranes adhered to the benchtop and fast transfer to the blocking solution was not possible. It was also important to see where the clear solution containing the UV-DNA was spotted, in order to prevent blotting of antigen more than once on the same location of the membrane. For these reasons, several methods of blotting were tried before a final one was adopted.

Initially the membranes were transferred to a piece of precut perspex which allowed 6 to 9 membranes to be laid out on top of the perspex and hence blotted together. Underneath the perspex a piece of card with the outline of equally spaced circles was placed. This allowed equally spaced blots to be blotted. When the antigen was blotted and the membranes allowed to dry, the membranes themselves became very dry (without the blots drying). This was unsatisfactory because it led to patchiness of the blot when visualised on the luminescence film. Thus a new technique had to be developed.

ELISA plates offer an alternative means of blotting. The plate have a 12 x 8 well configuration (see Figure 5.3a.). Four adjacent wells were overlaid with a single membrane, which was positioned over the wells with tweezers. Because

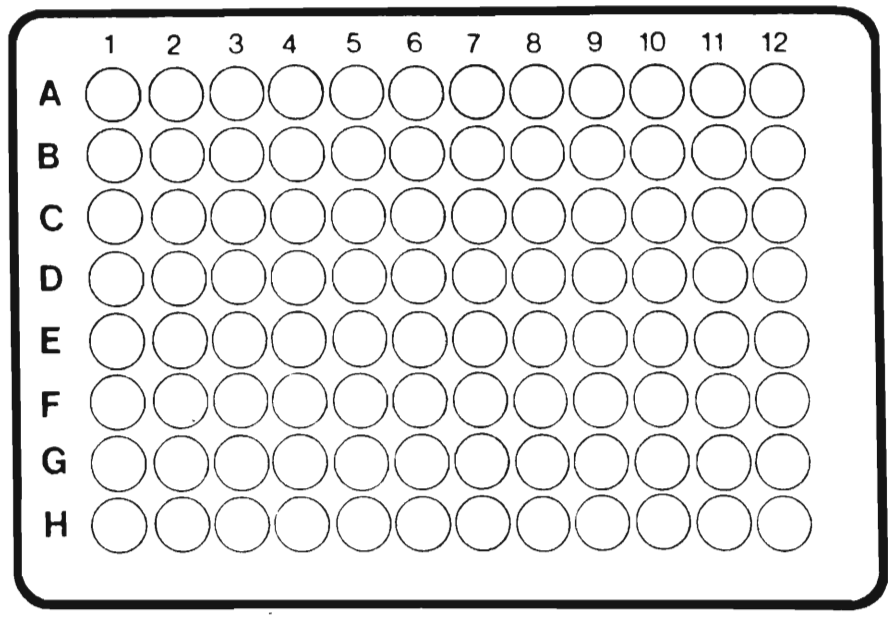


Figure 5.3a. An ELISA plate (96 well configuration).

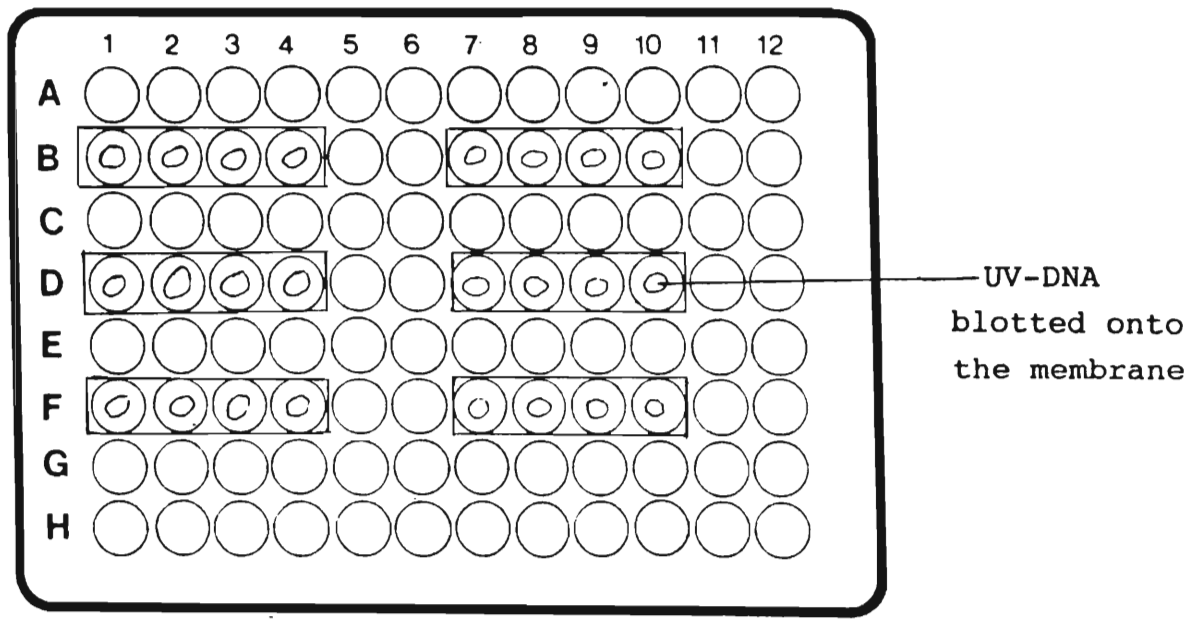


Figure 5.3b. An ELISA plate overlaid with pre-cut Immobilon-N membranes and blotted with the UV-DNA antigen.

the membranes were now exposed to air on both surfaces, the blots should have been able to dry more efficiently in comparison with drying on a perspex plate. The ELISA plate also helped in locating where antigen had been spotted (see Figure 5.3b). Although the ELISA plate was an improvement over blotting on perspex, the blots still took too long to dry (approximately 30 minutes). More efficient air flow across the membranes was required, and this was achieved by removing the base of each flat bottomed well and placing the ELISA plate across the top of an open plastic box. Using this crude technique, each membrane was positioned across 4 adjacent open wells of an ELISA plate, and the blotting performed while the ELISA plate was positioned over the open plastic box. The blots dried within 15 minutes which was insufficient time for the membranes themselves to dry. If this technique had not worked, it would have been necessary to attach a vacuum system to the ELISA plate and forcibly dry the membranes by suction. This would, of course, have been an extremely harsh means of drying the blots and could have interfered with the delicate antigen/antibody interactions on the membrane surface.

The pre-wetted membranes were thus individually transferred from the PBS buffer using tweezers. To facilitate easy evaporation of the PBS buffer from the membrane, each membrane was shaken manually, holding the membrane with tweezers. With shaking, the membranes were dry within 30 seconds after transferral. The membranes were then placed on the modified ELISA plate. The letter or number of each membrane, located on the top left hand corner of the membrane, faced upward on the ELISA plate. The ELISA plate was then placed over an open plastic box ready for blotting of the UV-DNA antigen.

As was outlined in Section 4.2.1.3, the UV-DNA antigen was heat denatured before being complexed to MBSA and Freunds

Incomplete Adjuvant. This important procedure ensured strand separation of the individual strands of the DNA antigen and thus facilitated maximum exposure of the immunogen (or antigen) to the rabbit antibody producing cells. Briefly, the denaturation involved heating the UV-DNA solution (contained in a Durham tube) at 100°C on a PEG bath for 10 minutes. The sample was then rapidly transferred to a beaker containing crushed ice for a further 10 minutes. The latter treatment ensured strand separation of the double helical DNA after the strands had been initially separated by heating.

The immunogen used for rabbit immunizations consisted of a complex mixture of DNA, MBSA, Freunds Adjuvant, some acetophenone (to ensure photosensitized production of thymine dimers only) and a small amount (less than 3%) of associated histone protein dissolved, or partly dissolved, in PBS. Since all these components were injected into the rabbit, antibodies were produced against all of them, including the thymine dimer. For the quantitation only the antigen generated against thymine dimer was relevant, so it was important to exclude the addition of MBSA and Freunds Adjuvant to the denatured UV-DNA that was used in the immunoblotting protocol. The acetophenone was largely evaporated during denaturation and the histone protein was at a sufficiently low concentration to be ignored. Excluding the addition of MBSA and Freunds Adjuvant thus reduced the possibility of antibody generated against MBSA and Freunds Adjuvant responding to the UV-DNA antigen. As will be shown in Section 6.5, the antibodies produced by the rabbits against thymine dimer in DNA in this study, were only 40% specific for thymine dimer.

An important criterion in dot blotting protocols is the volume of denatured UV-DNA antigen that is blotted manually onto the membrane surface using a micropipette. The volume that is chosen, must dry on the membrane surface faster

than the membrane itself dries. If an excess of antigen is blotted, factors such as site restriction and stereochemical constraints might be important when the antigen containing membrane is incubated with antibody solutions and the antibody might not be able to interact with the full complement of antigen presented on the membrane surface.

A literature search revealed that the majority of immunoblot protocols used commercial blotting apparatus (e.g. a 24 well Hybri-slot manifold, as used by Wani et al.[162]) which dried the blots under a low vacuum. This technique delivers a constant volume of antigen to the membrane surface, as well as ensures a constant shape (e.g. 0.5 x 4 mm^2) of the blot. This latter feature is important at the detection stage if luminescence film containing the the antigen/antibody complexes is to be quantitated by densitometric analysis of the blots. Here the intensity or darkness of the blot on the luminescence film is proportional to the amount of antigen present on the membrane (see Section 6.3.2).

In this work, a commercial blotting apparatus was available, but prohibitively expensive. Hence, a Volac 10-50 μl micropipette was used to deliver a constant volume of antigen to the membrane surface. Initially 4 x 10 μl aliquots of the heat denatured UV-DNA antigen were delivered to 4 individual sites in the membrane surface located over the wells of the modified ELISA plate. However, this volume of antigen was too large and the individual antigen blots spread into one another on the membrane. Hawkes et al.[169] blotted samples as large as 20 μl, and also suggested the use of a Hamilton syringe to deliver volumes as small as 100 nl of antigen to the membrane surface. If such a small volume was chosen, the antigen sample would have to be very concentrated to detect the blot on the luminescence film. Because the UV-DNA

antigen used in this work was 1 mg/ml or less in concentration, a volume substantially larger than 100 nl was used. Hence a volume of 5 μl was chosen, and this volume of antigen was delivered to the membrane surface using a 2-10 μl positive displacement Gilson micropipette. A protocol was tested using this volume of delivered antigen. The blots were easily visualized on the luminescence film, and hence this volume of antigen was chosen for the immunoblotting protocol.

It was important to keep the micropipette in an upright position whilst pipetting so that an even coverage of the membrane was achieved. It was also important not to scratch the surface of the membrane with the micropipette tip so that scratch marks did not appear on the luminescence film.

Usually between 12 to 18 membranes were treated during a single protocol, and thus the membranes were transferred from the PBS solution to each of 2 ELISA plates (6 to 9 membranes per plate). After blotting the membranes, the blots were allowed to dry by evaporation. By holding the ELISA plate (containing the blotted membranes) to the light, it was easy to identify when a blot had dried. When all blots (usually 3 to 4) on a single membrane had dried, the membrane was transferred with tweezers to the blocking solution contained in a plastic box. This was continued until all membranes from the same ELISA plate had dried, and eventually each of the two plastic boxes contained between 6 and 9 membranes each in the blocking solution (2% dried milk in 0.1% PBS-T).

At this stage, Millipore suggested an additional step in the protocol which allows for the fixing of the antigen to the membrane by means of baking or UV-crosslinking. It must however be kept in mind that the capacity and retention of nucleic acids on a membrane surface may be affected by the method used to treat the membrane subsequent to blotting

with the antigen. Fixing the blots by means of baking at 80°C in an oven for 1 to 2 hours was recommended by Millipore and this has been shown to increase the retention of nucleic acids on the membrane by 10 to 30%. An alternative technique which could also be employed involved the exposure of dried membranes to a UV source (312 nm transilluminator) for 3 to 5 minutes, after the which the membranes were re-wet in 100% methanol.

Seeing that oven baking the membranes increased an already lengthy protocol (24 to 26 hours) by an additional 2 hours, the latter UV crosslinking fixing step was adopted in the developmental stages of the immunoblotting protocol. This was performed by exposing the membranes individually to an HBO light source (as described in Section 2.8.1.1) with an attached 313 nm filter for three minutes. It was found that the membranes became extremely dry and white after UV-crosslinking. In addition, it was not a satisfactory technique to expose the antigen to a further UV dose, since a small amount of thymine dimer could well have been generated during this time. This was especially important when a comparison of the immunoblotting technique versus the HPLC technique was performed using the same UV-irradiated DNA sample for analysis (see Section 6.3). When the final immunoblotting protocol for the detection of thymine dimer in DNA was established, the technique of UV-DNA crosslinking was not used to fix the antigen to the membrane.

5.2.1.7.3 Membrane Blocking with 2.0% Dried Milk in 0.1% Tween-20 in PBS

As mentioned in Section 5.2.1.4, the blocking solution was 2.0%(w/v) dried milk powder in 0.1%(v/v) Tween-20 in PBS. The blocking solution was stored in the refrigerator, and when required, the solution was allowed to come to room temperature. A 20 ml aliquot, as suggested by Thomas[167],

was transferred to each plastic box using a beaker.

The boxes (containing 6 to 9 membranes each and the 2% dried milk solution) were sealed using lids, and each box placed on the shaking waterbath. During all processing stages (washing and incubation) the boxes were shaken horizontally at approximately 60 cycles per minute. The membranes were shaken for one hour during the blocking stage, as suggested by Amersham. This step served to block all sites on the membrane (where antigen had not been bound) with the dried milk solution.

5.2.1.7.4 Membrane Washing

When the blocking was complete, the boxes were removed from the shaking waterbath and the dried milk solution decanted, leaving the membranes in the box. A 10 ml aliquot of wash solution (0.1% PBS-T) was added to each box using a 10 ml beaker. Any membranes which adhered to the edges of the box were manouvered to the base of the box, and the boxes shaken vigorously by hand for 1 minute on a benchtop. This vigorous wash dislodged any excess blocking solution from the membrane surface or from the interior of the box. The wash solution was decanted, and the vigorous washing routine repeated. After decanting this second wash solution, another 10 ml aliquot of wash solution was added to each box, the boxes were sealed, transferred to the shaking waterbath and washed for 15 minutes.

When this 15 minute wash was complete, the lids of the boxes were removed, and the wash solution decanted. Any membranes adhering to the edges of the box were again manoeuvered to the base of the box and a fresh 10 ml aliquot of wash solution was added to each box using a 10 ml beaker. The membranes were washed for two successive 5 minute washing routines on the shaking waterbath, with fresh wash solution added after the first 5 minute wash.

Hawkes et al.[169] does not suggest a washing step after blocking. Instead, the blots were incubated in crude serum before being washed and then blocked a second time. Since a single blocking step produced a satisfactorily low background in this work, a second blocking stage was omitted in this work.

The details of washing routines were not specified by the workers using an immunoblot technique for the quantitation of modified DNA constituents[84,161,162]. Hence the washing routine as specified by Amersham was used.

To summarize the immunoblotting protocol thus far:
Immobilon-N membranes were cut and pre-wet in 100% methanol, water and PBS. Individual membranes were transferred to a modified ELISA plate. The membranes were blotted with 5 μl aliquots of denatured UV-DNA applied to the surface of the membrane using a micropipette. The membranes were blocked with a 2% dried milk in 0.1% PBS-T solution for 1 hour on a shaking waterbath. The membranes were then washed by means of:

a) two brief, but vigorous 1 minute washings,
b) one 15 minute wash, and
c) two five minute washes.

After an extensive wash routine which removed excess blocking solution from the membrane surface, the membrane had only antigen bound at 3 to 4 locations along its surface. Hence all other sites on the membrane were blocked with blocking solution only. This blocking regime ensured that antibody present in the crude serum would only bind to antigen, since specific antigenic determinants (largely thymine dimers) of the antigen would respond to specific binding sites of the antibody.

5.2.1.7.5 Incubation with Dilutions of Crude Rabbit Serum

Rabbit blood was obtained while bleeding the rabbit from the marginal ear vein (see Section 4.2.2.4). The blood was spun down with a centrifuge, thus separating the crude serum (containing the antibodies) from the red blood cells which were discarded. Several immunoblotting runs were performed using the crude serum and because the antigen/antibody was easily visualised on the luminescence film during detection, the protocol was performed on the crude serum without using purified IgG. An affinity chromatography technique was used in this work to purify the serum (see Sections 4.2.3.4 to 4.3.2.10). Only IgG, and not IgM, was separated from the serum proteins in this routine. An alternative technique of ammonium sulphate precipitation of the antibody was also attempted (see Sections 4.2.3.1 to 4.2.3.3). This technique was successful in purifying antibody in the crude serum, but because of the associated problems of only being able to purify less than 500 μl of serum obtained from a bleed of the rabbit (Section 6.1.1) crude serum was used for all immunoblotting protocols.

As outlined in Section 4.2.2.4, 1.5 ml aliquots of serum was stored in Eppendorfs in the freezer. Before the serum was used it was removed from the freezer and allowed to reach room temperature.

For this work, quantitation of the antibody against the antigen was performed using immunoblotting with enhanced chemiluminescence in order that antibody production as a function of time could be determined. This would give an indication when to bleed the rabbit, in order that the maximum amount of antibody could be obtained for use in any subsequent analysis stages of the immunoassay protocol. To this end, the crude serum was diluted (see later) and the protocol performed using the same antigen concentration and

a range of antibody dilutions. The more antibody present in the serum, the further the serum could be diluted, until the antibody was at such a low concentration that it could not bind to the antigen to give detectable blots on the luminescence film. Thus the more a serum was diluted, the higher the concentration of antibody present in the serum. As explained by Fonong et al.[166], the highest dilution of antibody that yields a visible blot on the luminescence film defines the end point or titre of that serum. In this manner, a relationship between antibody production as a function of time was obtained.

Rabbits were bled at weekly intervals for 4 weeks, followed by blood being drawn twice at 10 day intervals. It is likely that antibody production increases as a function of time, and to determine the highest antibody titre for an overall immunization schedule (4 injections and 6 bleeds), the antibody titre of the serum obtained from bleed 6 was determined.

The serum was diluted by serial dilution. First, 100 μl of serum was removed using a Volac 20-200 μl micropipette and transferred to a clean and dry 12 ml glass vial (Number 3 from Polytop). Then 4900 μl of the wash solution/diluent (PBS-T) was added using a Volac 100-1000 μl micropipette to give a total volume of 5000 μl or 5 ml. It was important that any diluent adhering to the inner walls of the pipette tip be allowed to run to the exit of the tip, so that the entire volume could be dispensed. The vial was sealed and shaken for a few seconds by hand to disperse the serum in the diluent. Thus a 1:50 dilution of the crude serum was prepared. The vial was labelled with the dilution and date to facilitate identification.

To prevent contamination between successive dilutions, a new pipette tip was used to withdraw 2500 μl of the 1:50 dilution. The solution held in the pipette tip was then

transferred to a clean and dry 12 ml glass vial. A new pipette tip was then used to add 2500 µl of the PBS-T diluent to the second glass vial. The vial was sealed and shaken as before. Serial dilution of the 1:50 dilution thus yielded a 1:100 dilution of the second sample.

Using this serial dilution technique, the following dilutions were prepared:

Remove 100 µl serum + 4900 µl PBS-T	1:50
Remove 2500 µl of above + 2500 µl PBS-T	1:100
Remove 2500 µl of above + 2500 µl PBS-T	1:200
Remove 2500 µl of above + 2500 µl PBS-T	1:400
Remove 2500 µl of above + 2500 µl PBS-T	1:800
Remove 3333 µl of above + 1667 µl PBS-T	1:1200
Remove 2500 µl of above + 2500 µl PST-T	1:2400
Remove 3333 µl of above + 1667 µl PBS-T	1:3600
Remove 3333 µl of above + 1667 µl PBS-T	1:5400
Remove 3333 µl of above + 1667 µl PBS-T	1:8100
Remove 3333 µl of above + 1667 µl PBS-T	1:12150
Remove 3333 µl of above + 1667 µl PBS-T	1:15187
Remove 3333 µl of above + 1667 µl PBS-T	1:18984

Once the dilution schedule was complete, the final vial contained 5000 µl of diluted serum. In addition, some vials contained 2500 µl of diluted serum, while others contained 1667 µl of diluted serum. Using a fresh pipette tip each time, a volume of diluted serum was withdrawn from each vial so that the final volume contained in each vial was 1667 µl.

Although the list is not exhaustive in terms of all the dilutions that were performed, it does provide an indication of the procedures required in order to determine the antibody titre of a single serum sample.

As mentioned earlier (Section 5.2.1.7.2), 12 to 18 membranes were treated in a single protocol. Seeing that 2

rabbits received the same immunogen via a subcutaneous or intramuscular injection route, it was usual to determine the antibody titre of a particular serum sample (e.g. 5th bleed) for the subcutaneous rabbit and the intramuscular rabbit simaltaneously. Thus 6 to 9 membranes were spotted with the antigen (e.g. 0.5 mg/ml denatured and UV-irradiated DNA) and then subjected to 6 to 9 dilutions of the subcutaneous serum antibodies, while the other 6 to 9 membranes were spotted with the same antigen and subjected to 6 to 9 dilutions of the intramuscular serum.

To perform the antibody incubation of the membranes, a single membrane (4 x 1 cm^2) was removed from the wash solution and transferred to a glass vial containing 1667 μl of a specific antibody dilution. The dimensions of each membrane were carefully chosen so that the membrane could be accommodated within the glass vial. Each membrane was labelled 1,2,3 or A,B,C, etc. and the surface of the membrane containing this number and the blotted antigen, was transferred to the vial in such a manner that the antigen containing surface faced the outside of the vial. This ensured that the antigen bound membrane surface was always in contact with the diluted serum while lying horizontally throughout the incubation period. Numbering or lettering of the membrane was also important so that a particular membrane could be correlated to a specific antibody dilution. To this end, a comprehensive list was compiled of membrane number, antigen concentration spotted and the corresponding antibody dilutions used in a particular protocol. This list prove invaluable when analysing the luminescence film for the cut-off antibody dilution.

Once the membranes were transferred using tweezers to their respective glass vials, the vials were transferred to the same clean and dry plastic boxes used for blocking and washing. The two boxes were sealed with lids and then

transferred to the shaking waterbath.

Initially the membranes were incubated in the antibody dilutions for an hour, according to Amersham's instructions. However this period of incubation was far too short as no blots were detected on the luminescence film. The protocol was thus tailored to incubate the membranes overnight for a 14 to 16 hour period. This incubation time allowed the antibody to bind exclusively with the antigen on the membrane surface, and the blot was successfully detected on the luminescence film. Hawkes et al.[169] suggested a 2 to 4 hour incubation period for dilute solutions. However an overnight incubation was shown to be as much as ten times more sensitive in immunoblotting protocols carried out here. Hence an overnight incubation period was used in all subsequent protocols.

5.2.1.7.6 Membrane Washing

Once incubation with the crude serum dilutions was complete, the blots were again washed to remove an excess of unbound antibody from the membrane surface. This was performed by removing the plastic boxes from the shaking waterbath and removing the membranes from the vials. All membranes incubated with the dilution from the same serum (e.g. subcutaneous, bleed 5) were transferred to another plastic box containing 10 ml of wash solution, PBS-T. The membranes that were incubated in the intramuscular diluted serum were transferred to a second plastic box containing 10 ml of wash solution. As outlined in the previous washing routine (Section 5.2.1.7.4), the membranes were subject to 2 vigorous washes, a single 15 minute wash and two 5 minute washes.

At the completion of the wash routine, each blot on a membrane consisted of UV-DNA antigen and antibody directed against the antigen. Of course, antibodies were produced

against MBSA and Freunds Adjuvant, and because these antibodies would share similar, but not the same, antibody binding sites, they will also have bound randomly to the UV-DNA antigen.

5.2.1.7.7 Incubation with a Secondary Rabbit Antibody Conjugated to Horseradish Peroxidase

In order to quantitate the antigen/antibody complex, the final stage in the immunoblotting protocol required that a second antibody conjugated to an enzyme be bound to the antigen/antibody complex. As discussed in Section 5.2.1.5., this antibody enzyme system was supplied by Amersham as " Anti-rabbit Ig, horseradish peroxidase linked whole AB (from donkey)". The peroxidase enzyme was chosen because of its wide application in immunology; it can catalyse reactions where light is emitted (as in this work), or it can catalyse the production of a chromogenic reaction product, especially in ELISA applications (Leipold et al.[82]). Hawkes et al.[169] also specified HRP-conjugated antibodies for the detection of monoclonal antibodies and a range of soluble proteins, nucleic acids, bacteria and viruses.

The antibody/enzyme conjugate was stored in a refrigerator and was allowed to come to room temperature before use. Initially a 5 μl aliquot of the stock conjugate was blotted onto the membrane surface using a 2 to 10 μl Gilson micropipette and the membrane allowed to dry. When the blots were quantitated using enhanced chemiluminescence, no gradations of blot intensity as a function of antibody dilutions could be discerned. Hence the protocol was adapted to allow for incubation of the membranes in diluted conjugate solutions.

It was important to determine the optimum dilution of the secondary antibody/enzyme system. This would ensure maximum

binding of the conjugate to the immune complex localized on the membrane, while simultaneously reducing binding of the conjugate to the membrane itself. This optimization thus facilitated quantitation if the immune complex against a clear background on the luminescence film.

Here, a series of secondary antibody/enzyme dilutions were prepared in 12 ml glass vials (Number 3 from Polytop). A 1:100 dilution was prepared by delivering 25 μl of the conjugate to each of 18 glass vials using a 20 to 200 μl Volac micropipette. Each solution was made up to 2500 μl by addition of 2475 μl of diluent (PBS-T). The vials were sealed and the solution shaken to disperse the conjugate. An entire immunoblotting protocol was performed using this dilution of conjugate. A 1:500 dilution of conjugate was prepared by delivering 5 μl of the antibody/enzyme to each of 18 glass vials. The solution was made up to 2500 μl by addition of 2495 μl of PBS-T. The vials were sealed and an immunoblotting protocol performed. A final secondary antibody dilution was prepared by delivering 2.5 μl of the conjugate to each of 18 clean and dry glass vials using a 2 to 10 μl Gilson micropipette. This gave a 1:1000 dilution which was made up to 2500 μl in each case by addition of 2497.5 μl of PBS-T. The entire immunoblotting protocol was performed using the same antigen and primary antibody dilutions, with the 1:500 and 1:1000 dilutions of the secondary antibody.

Once the luminescence films were developed and printed, the blots on the film were observed to see which secondary antibody dilution gave the darkest blot on the film against the lightest background. For this work, the conjugate diluted 1:500 was chosen and used in all subsequent immunoblotting protocols. Thus eighteen 1:500 dilutions of the secondary antibody/enzyme solution were prepared in glass vials as outlined above for each protocol carried out.

To summarize, at this stage of the immunoblotting protocol, the membranes were blotted with antigen, washed in PBS-T, incubated in crude serum dilutions and washed again in PBS-T. Using tweezers, the membranes were individually removed from the plastic box where they had been washed, and each transferred to a glass vial containing a diluted secondary antibody solution. Unlike incubation of the membranes in the crude serum dilutions where each membrane was incubated in a different serum dilution, all secondary antibody dilutions were identical and hence any membrane could be transferred to any diluted conjugate solution.

The protocol as specified by Amersham allowed for a one hour incubation period. However once a protocol had been performed using this incubation period, it was decided to extend the incubation time to six hours. Because this longer incubation period allowed more secondary antibody/enzyme complex to bind to the antigen/antibody complex, the blot was more easily identifiable on the luminescence film. Hence 6 hours was chosen as the incubation period for secondary antibody/enzyme for all subsequent immunoblotting protocols.

Incubation with secondary antibody was performed by transferring the membranes with tweezers to individual glass vials and ensuring that the antigen/antibody bound surface faced the outside of the vial. The vials were sealed and if more than 9 membranes were being processed at a time, the vials were placed in 2 plastic boxes. The boxes were sealed and then transferred to the shaking waterbath set at approximately 60 cycles per minute for a 6 hour period.

5.2.1.7.8 Membrane Washing

Once the incubation in secondary antibody was complete, the final washing of the membranes took place. The boxes were

removed from the shaking waterbath, the vials removed from the boxes and the membranes transferred from the vials to clean plastic boxes to each of which had been added 10 ml of wash solution using a 10 ml beaker. Using the procedure outlined for all other washing routines, the membranes were vigorously washed thrice. This was followed by a single 20 minute wash on the shaking waterbath and four subsequent 5 minutes washes also performed on the shaking waterbath.

The immunoblotting protocol which was described above has allowed antigen, antibody and a secondary antibody conjugated to HRP to form a single immune complex immobilized on a membrane surface. The following section is a summary of the immunoblotting protocol which was finally adopted.

5.2.1.8 Summary of the Immunoblotting Protocol for Thymine Dimer Detection in UV-Irradiated DNA

1. Membranes were cut into 4 x 1 cm^2 strips and each strip labelled in the top left hand corner.
2. Membranes were pre-wet in
a) 100% methanol for 2 seconds,
b) Milli-Q water for 1 to 2 minutes, and
c) PBS for 15 to 30 minutes.
3. Membranes were transferred to a modified ELISA plate, allowed to dry, and then blotted with 5 μl aliquots of denatured UV-irradiated DNA.
4. The membranes were blocked in 2% dried milk in 0.1% Tween-20 in PBS for one hour on a shaking waterbath.
5. The membranes were washed using 0.1% Tween-20 in PBS as follows:
a) two brief, but vigorous washes,
b) one 15 minute wash, and
c) two 5 minute washes.
6. The membranes were incubated overnight in crude serum dilutions on the shaking waterbath.

7. The membranes were washed using 0.1% Tween-20 in PBS as follows:
a) two brief, but vigorous washes,
b) one 15 minute wash, and
c) two 5 minute washes.
8. The membranes were incubated for 6 hours in a 1:500 dilution of secondary antibody conjugated to horseradish peroxidase.
9. The membranes were washed using 0.1% Tween-20 in PBS as follows:
a) three brief, but vigorous washes,
b) one 20 minute wash, and
c) four 5 minute washes.

The relevant detection protocol (enhanced chemiluminescence, in this work) could be carried out as soon as the immunoblotting protocol was complete. If the detection protocol was not to be carried out immediately, the membranes were stored in PBS buffer in plastic boxes in the refrigerator.

The following section (5.2.2) describes the chemiluminescent protocol that was chosen for detecting and quantitating thymine dimer in UV-irradiated DNA. First, the phenomenon of chemiluminescence will be dicussed. Then, the choice of equipment used to perform the protocol will be discussed. Finally, a description will be given of the development and data collection stages of the chemiluminescent protocol.

5.2.2 VISUALIZATION AND QUANTITATION OF IMMUNE COMPLEXES USING ENHANCED CHEMILUMINESCENCE

The previous section (5.2.1) described the immunoblotting protocol that was developed in this work for the quantitation of thymine dimers in UV-irradiated DNA. This technique involved blotting the UV-DNA antigen onto

Immobilon-N charge modified membranes. Then the membranes were incubated in dilutions of crude serum (containing antibodies generated against the antigen). This allowed the antibody to bind to the antigen. The final step in the protocol involved incubation of the membrane in a dilution of the secondary antibody (specific for the first antibody). This secondary antibody was conjugated to the enzyme horseradish peroxidase, and it also bound to the immobilized antigen/antibody complexes. Thus immune complexes consisting of antigen, antibody and secondary antibody conjugated to HRP were immobilized on the membrane.

This section now describes the technique of enhanced chemiluminescence that was employed for the detection and quantitation of thymine dimer antigen immobilized on the Immobilon-N membrane.

5.2.2.1 Description of Enhanced Chemiluminescence as a Visualization Technique

Chemiluminescence may simply be described as the chemical production of light. This chemiluminescent process is said to occur whenever a molecule emits a photon of energy, as a result of an exothermic reaction in which one of the intermediate or end products is left in an electronically excited state. The chemiluminescent reaction occurs without the absorption of light and it has been found that approximately 200 kJ of energy is necessary to emit a photon[173]. In addition most chemiluminescent reactions have been found to be of the oxidative type where only the singlet state of the reactant species (luminol) is involved.

If the chemiluminescent process occurs in a biological system, then the process is called bioluminescence. This naturally occurring phenomenon was originally noted by

Aristotle as the glow of dead fish and fungi. Several other bioluminescent phenomena are well known, amongst them the light of glow worms and the light of some bacterial species. In contrast to bioluminescence, the chemiluminescent properties of luminol (a cyclic diacylhydrazide) and lucigenin were only discovered in 1928 and 1935 respectively, and their use in analytical and clinical chemistry noted decades later.

The use of chemiluminescence in chemical and clinical applications has led to investigations of the reactions and mechanisms involved. For luminol, the most widely used chemiluminescent compound in chemical assays, the light emitting reaction can be seen in Figure 5.4. White and Bursey[174] have demonstrated that the aminophthalate dianion is the light emitting species in the reaction. In general, electron donating substituents increase the efficiency of the chemiluminescent emission. To this end, di- and tri-substituted luminol compounds have been shown to be 13 and 30% respectively more efficient light emitters than luminol.

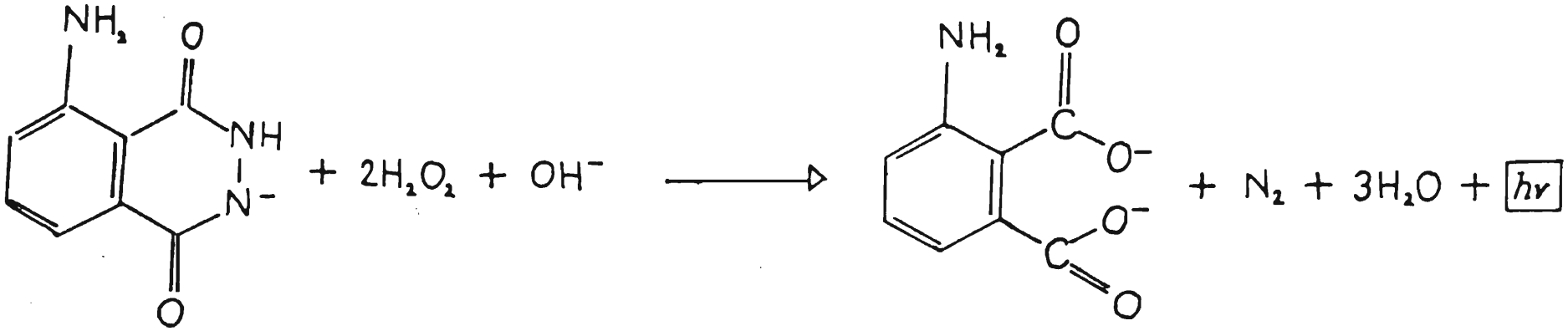
The mechanism of organic chemiluminescence in solution involves three key stages:

a) Preliminary reactions to provide the key intermediate.

b) An excitation step in which the chemical energy of the key intermediate is converted into electronic excitation energy.

c) Emission of light from the excited product formed during the course of the reaction.

The precise mechanism of formation of excited species from ground state molecules in solution at room temperature is unknown. The only clue to resolving the mechanism is that oxygen is involved in most, if not all, chemiluminescent reactions. It has been proposed that oxygen is in its



luminol mono-anion

aminophthalate dianion

Figure 5.4. Schematic diagram representing a chemiluminescent reaction involving the oxidation of luminol to produce light of wavelength 425 nm to 430 nm.

triplet ground state, hence oxygen may exist as triplet state complexes. Loss of nitrogen from the oxygen adduct (or the gain of energy by some other process) could lead to a vibrationally excited triplet state of the emitter species. The excited triplet species could then inter-system cross to its excited singlet state, after which chemiluminescence is effected by the emission of a photon of light of between 425 to 430 nm from the excited species (see Figure 5.5.).

The efficiency of this light emission process varies according to the reactant species involved. To this end, the quantum yield of a chemiluminescent reaction is defined as the number of photons of energy emitted during the reaction, versus the number of molecules involved in the specific luminescence reaction. For most chemiluminescent systems this quantum yield is seldom greater than 0.01, while bioluminescent reactions have quantum yields approaching unity. The quantum yield for luminol ranges from 0.01 to 0.05[175], while measurements in dimethyl sulphoxide have yielded an answer of 0.05 - the highest quantum yield for chemiluminescent compounds[174]. These figures suggest that the bioluminescence process is a more efficient energy releasing process than chemiluminescence. Despite the low quantum yields for chemiluminescent systems, compounds which are chemiluminescent by nature have been used in several analytical and clinical applications[176]. Auses et al.[177] used a luminol-ferricyanide-hydrogen peroxide system to determine the glucose levels in human serum. Gorus et al.[178] determined uric acid concentrations using the enzyme uricase, to which the addition of a luminol-potassium ferricyanide-hydrogen peroxide system allowed chemiluminescent detection and quantitation of uric acid. Other substrates also produce light upon reaction with hydrogen peroxide. One such example is TCPO [bis(2,4,6-trichlorophenyl) oxalate] which has been used instead of luminol by Williams and Seitz[179]

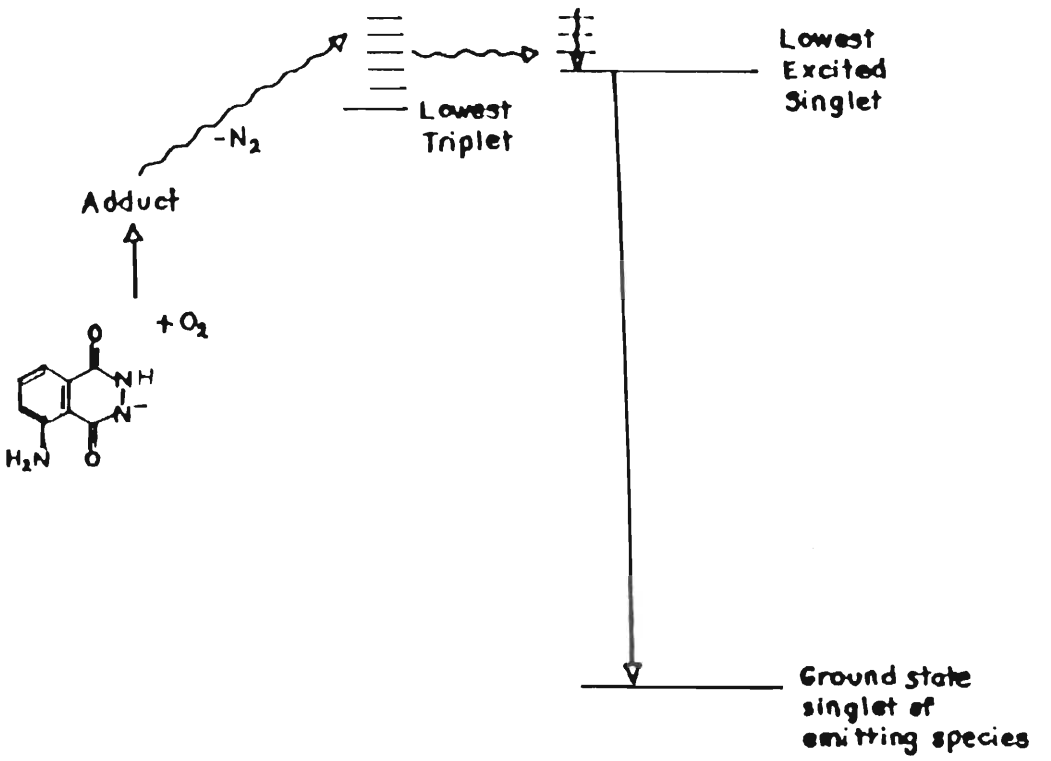


Figure 5.5. Diagram representing the mechanism of organic chemiluminescence[174].

to assay for NADH and the enzyme lactate dehydrogenase.

In addition, several chemiluminescent systems have been used to monitor immunological reactions. The majority of these utilize luminol or luminol derivatives such as isoluminol (6-amino-2,3-dihydrophthalazine-1,4-dione) and o- and m-aminobenzhydrazides in the immunoassay.

Amongst other applications, immunological assays have allowed small numbers of micro-organisms to be rapidly identified and quantitated using antibodies linked to the enzyme, horseradish peroxidase. In the presence of pyrogallol and hydrogen peroxide, light emission catalysed by this enzyme allowed as few as 30 bacterial cells to be detected[180]. Schroeder et al.[181] have used an immunochemiluminometric assay for the quantitation of hepatitis B surface antigen. Chemiluminescence has also been used as the means of quantitation of extremely low levels (femtogram amounts) of the peroxidase enzyme when a luciferin derivative was used as the substrate. This approach has been applied to the estimation of peroxidase-coupled antibodies bound to mouse lymphocytes[182].

These luminescent enzyme immunoassays have the advantages of cheap and stable reagents, the assays are rapid, and when used in a clinical laboratory, the assays can be automated. In addition, the radiation hazards associated with radioimmunoassays are obviated and chemiluminescent measurements are easy to make and require no complicated apparatus. However, the most important advantage for the work presented here, is the sensitivity of the detection system. Detection limits for the luminescent assay of ATP and NADH are reported to be 10^{-10} mole and 10^{-16} mole respectively[178]. Luminescent assays are, in addition, more sensitive than the conventional colorimetric assays (ELISA) that can be used. This sensitivity feature has been exploited in the quantitation of very low levels of analyte

or sample.

Since chemiluminescent assays appear to provide the sensitivity required in this work, it was decided to apply a chemiluminescent substrate to the thymine dimer containing immune complex localized on the Immobilon-N membrane. Commercial manufacturers claimed that the chemiluminescent assay could detect picograms of antigen localized on blots. Seeing that these levels fell below the 2.5×10^{-6} M concentration of dimer detectable by HPLC, the chemiluminescence quantitation technique appeared to provide a viable means of quantitation of thymine dimer.

To improve an already sensitive technique, Whitehead et al.[184] in 1984, developed a procedure of enhanced chemiluminescence for the sensitive determination of peroxidase-labelled conjugates in immunoassays. Their work proposed that the simultaneous use of synthetic firefly d-luciferin and luminol, or a luminol analogue, in the presence of an oxidant, allowed for an 80-fold enhancement of signal-to-background ratio in an immunoassay protocol. Using this synergistic approach, total light emission from the luciferin and luminol analogue together, significantly exceeded that from either compound when used alone (Figure 5.6.). This technique has successfully been implemented for the immunoassay of serum α-fetoprotein, IgE and rubella virus antibody.

This pioneering research has led to the development of enhanced chemiluminescence (ECL), that is, the sustained emission of light, provided by the inclusion of chemical enhancers in the HRP catalysed oxidation of luminol. With the addition of suitable enhancers, this technique gives about 10 to 100 times more light than oxidation of luminol alone. These enhancers do not increase the quantum yield of the reaction, but do regulate the reaction between HRP, oxidant and luminol. Enhanced systems have also been shown

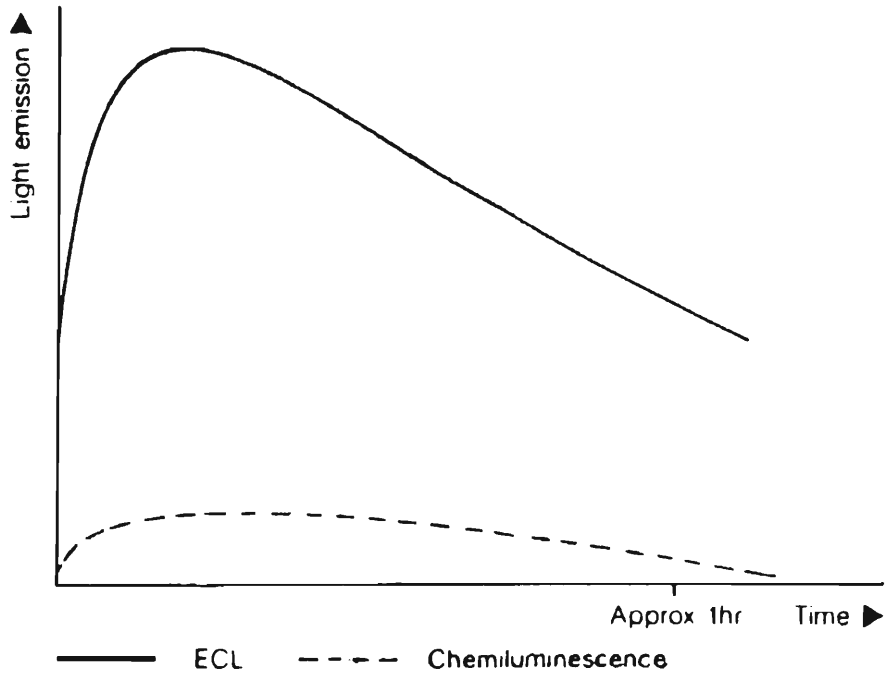


Figure 5.6. Graph illustrating the qualitative difference between chemiluminescence and enhanced chemiluminescence.

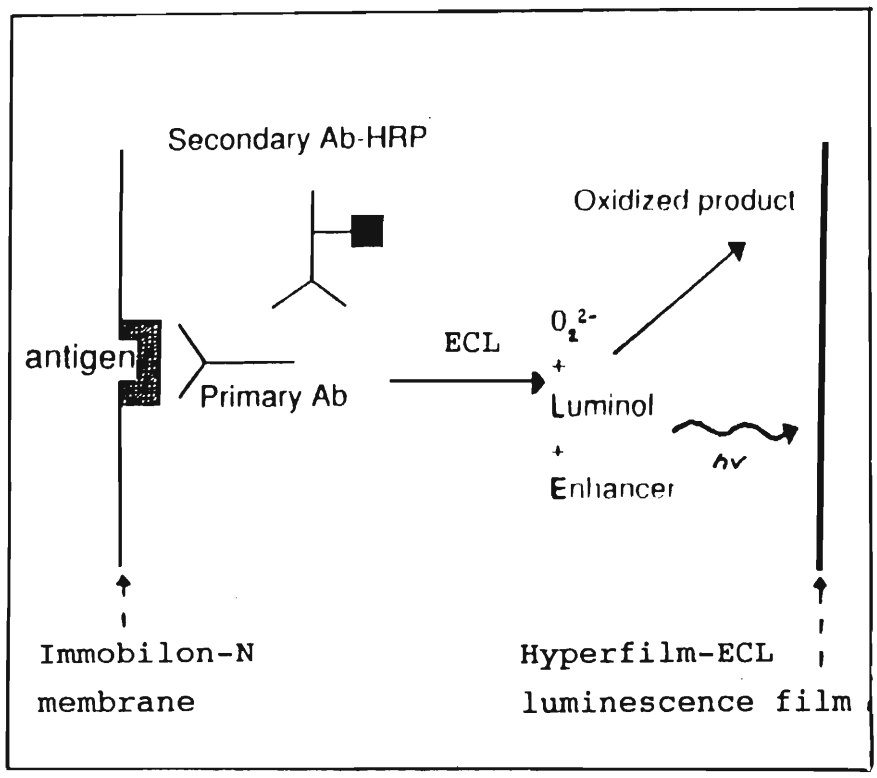


Figure 5.7. Diagram representing the principles of dot blotting used in conjunction with enhanced chemiluminescence.

to depress the light emission in the absence of HRP thus contributing to an improved signal-to-background ratio. The light generated in enhanced chemiluminescence is emitted within 30 seconds, in comparison with colorimetric assays which can take 30 minutes for development. These enhanced light emissions are sufficiently intense to impinge on high speed Polariod instant film exposed to the luminescing compound. This observation was noted by Thorpe et al.[185] in 1984 who reported the use of high speed instant photographic film for the quantitation of serum ferritin in an enhanced luminescent immunoassay.

Previously, photographic monitoring of luminescent immunoassays was not possible because the low light intensities generated were not easily detected, even with highly sensitive film. In contrast, light emission from the luciferin enhanced chemiluminescent reaction of luminol with HRP conjugates, was sufficiently high to allow the photographic film to be used as a detector. In addition, the light emission has been shown to be constant over a period of minutes[185] and the luminescent reaction can be initiated before exposure of the membrane to the film itself. Photographic detection also has the advantages of requiring no external power source, the technique is rapid, and a permanent hard copy of the results is obtained.

In this work, (refer to Figure 5.7.), a luminol-hydrogen peroxide detection system was added to the immobilized immune complex on the Immobilon-N membrane. The enzyme, HRP, (conjugated to the secondary antibody) catalysed the conversion of luminol to the aminophthalate dianion. In the process, light of 425 to 430 nm was emitted. The intensity of the emitted blue light is proportional to the amount of HRP bound to the membrane which in turn is proportional to the amount of secondary antibody bound to primary antibody and this proportional to the amount of antigen bound directly to the membrane. Thus the emitted light intensity

can be used to quantitate the antigen bound to the membrane. When an X-ray or luminescence film (e.g. Hyperfilm-ECL) is superimposed on the membrane and luminol-hydrogen peroxide added to the complex, the light impinges on the film which can then be developed and printed to allow the intensity of the emitted light (and hence bound antigen) to be quantitated.

5.2.2.2 Enhanced Chemiluminescence Equipment and Operation

The enhanced chemiluminescent (ECL) detection system that was chosen was supplied by Amersham as an "ECL Western Blotting detection system" (Catalogue Number RPN 2106). The detection system was supplied as two 250 ml solutions stored in white and black polythene screw-top containers. These solutions were maintained at 2 to 8°C in the refrigerator.

As mentioned in the previous section, several luminescent substrates are commercially available, with luminol being the most widely used compound. In order to ensure that the chemiluminescent reaction proceeds optimally, an oxidant must be added to the system. Most common is the use of hydrogen peroxide, although hypochlorite, iodine and permanganate can also be employed[176]. Catalysts that are used in this luminescent reaction include $Fe(CN_6)^{3-}$ and Cu^{2+}, with the most efficient catalyst being heme. It is usual for the reaction to proceed in an oxidizing environment around pH 11.

The Amersham ECL detection solution 1 contained the substrate for the reaction supplied as a per-acid salt in a buffer solution at pH 9.5. Detection solution 2 contained the oxidizable chemiluminescent compound, luminol, which served to generate the light signal. In addition, an enhancer was supplied, but not specified, in this second

detection solution. This ensured that the chemiluminescent reaction generated a sustained enhanced light output. The solutions were provided at their working strength and were sufficient for the detection of 4000 cm^2 of membrane. This ECL detection system was suitable for detection of sub-picogram amounts of sample and to this end, was 10 times more sensitive than colorimetric (ELISA) assays.

As well as the detection solutions necessary for chemiluminescent immunodetection, other equipment is also required. A suitable X-ray film to capture the emitted light is necessary and was supplied by Amersham (Catalogue Number RPN 2103) as Hyperfilm-ECL. This is a high performance double sided luminescence film developed for the detection of light emitted in the course of ECL. It was important to store the film properly, away from X-rays, gamma-rays or other penetrating radiation. It was also recommended that the film be stored at 4°C. Thus it was stored in the refrigerator away from any chemicals or noxious fumes.

The film was only opened in the dark or under safelight illumination, and only prior to being used for assay purposes. When it was required, a single 18 x 24 cm^2 sheet was removed under a safelight and the sheet cut into approximately 8 x 4 cm^2 strips to cover the membranes during detection. This will, however be described in Section 5.2.3.2. No recommendations as to the stability of the film were given by the manufacturers, but it was found that after a 6 month period, the film no longer yielded a clear background when developed and printed without the light from chemiluminescent immune complexes impinging on it. The film was thus discarded and a new box acquired.

The ECL-Hyperfilm was specifically chosen because of its characteristics in detecting light emission during chemiluminescent assays. If the immune complexes were being

detected by a radioimmunoassay, then a multipurpose autoradiography film, a high resolution film for autoradiography or a single coated film for the direct autoradiography of tritium and ^{125}I labelled compounds would have been chosen.

In order that all stray light be excluded from the film while in contact with the Immobilon-N membrane, the film and membrane were housed in a Hypercassette during detection. The Hypercassette was supplied by Amersham (Catalogue Number RPN 1642) and was 18 x 24 cm^2 in area. The interior of the cassette was black and non-reflective for stray light absorption. The cassette was constructed from hard aluminium alloy which allowed use at room temperature or -80°C.

Hypercassettes are suitable for the quantitation of radiolabelled compounds using photographic detection, or for chemiluminescent applications. In order that the cassette remained in good working order, it was important to wipe the cassette out with paper towel after each use. The use of strong acid or alkali was not recommended because of the degradative properties of these chemicals.

When the cassette was being used in this work, the membranes were overlaid with detection solution for 1 minute and then sealed in cling film before being transferred to the cassette. In the dark, a strip of luminescence film was placed over the light emitting membranes, and the cassette closed during exposure of the light to the film. The film was then developed and printed using X-ray photographic chemicals. The entire ECL detection protocol will be explained in Sections 5.2.2.3.1 and 5.2.2.3.2.

Additional equipment required for this ECL protocol included blunt tip fine forceps for handling the membranes

and luminescence film, a timer with fluorescent dials that could be seen in the dark, and clingfilm to wrap the membranes during chemiluminescent detection. The supplier of the detection solution (Amersham) suggested the use of Saranwrap for the latter purpose. However the clingwrap available in this laboratory was adequate and was used throughout the developmental and data collection stages of the chemiluminescent protocol. The plastic boxes that were used during immunoblotting were also used during this protocol; during the detection stages and during photographic development and printing.

Standard X-ray developer and fixer were used to process the luminescence film. A Phenisol developer (5 litre) and Hypam fixer (5 litre) supplied by Ilford were used. These 5 litre solutions were stored in the dark and had to be diluted before use. The dilutions as suggested by Ilford were performed by transferring 100 ml of developer to a clean and dry 500 ml amber bottle using a 100 ml measuring cylinder. Using the same cylinder, 4 x 100 ml aliquots of Milli-Q water were added to the amber bottle. This gave a 1+4 dilution. The bottle was sealed and shaken to dissolve the developer.

In the same manner, a 50 ml measuring cylinder was used to transfer 50 ml of fixer to a clean and dry amber bottle. Then, 450 ml of Milli-Q water was added using the 100 ml cylinder. This gave a 1+9 dilution of the fixer. The bottle was sealed and shaken to dissolve the fixer. Both the diluted developer and fixer were stored in the refrigerator and allowed to come to room temperature before use. Seeing that the developer contained hydroquinone which is easily oxidized, the diluted developer had a limited shelf life. When it was freshly prepared, it was a very pale lemon colour. When the colour changed to a deep orange/brick red, the developer had expired and was not fit for use. It was thus discarded and prepared fresh. The fixer did not have

any oxidative properties and hence could be used for longer periods.

When these solutions were required, 200 ml of each was poured into individual plastic boxes (10 x 8 x 6 cm^3) into which the luminescence film was immersed. The film was first immersed in the developer, followed by immersion in the fixer. The photographic detection of immobilized immune complexes will be discussed in Section 5.2.2.3.2.

5.2.2.3 Development and Use of a Working Protocol

The previous section has described the reagents and equipment used in the development of a chemiluminescent protocol for the quantitation of thymine dimer in UV-irradiated DNA. This section now describes the usage of these chemicals and equipment, while the subsequent section discusses the photographic protocol that was adopted for the development and printing of luminescent film, in order that the immune complexes were vizualized on the film.

5.2.2.3.1 Development and Use of a Working Chemiluminescent Protocol

The protocol that was adopted for immunoblotting (Section 5.2.1.8) was specifically tailored to allow for optimum signal strength of the immune complex visualized on the luminescence film. In the same manner, the ECL protocol has also been developed to allow optimization of the immune signal during detection. In this way, the entire immunoassay has been tailored so as to allow detection and quantitation of very low concentrations of thymine dimer generated in low concentrations of UV-irradiated DNA.

At this stage of the assay, the Immobilon-N membranes contained bound immune complexes localized at several positions along their surfaces. In addition, all extraneous

serum components and proteins were largely prevented from binding to the membrane surface at locations where the immune complexes were not immobilized.

During the chemiluminescent protocol the use of gloves was strongly recommended to prevent hand contact on the film or detection reagents.

The membranes were removed from the PBS solution in the plastic box to a second clean and dry plastic box. It was important to place the membranes with their antigen/antibody surfaces facing upwards in order that the detection solution (containing luminol and hydrogen peroxide) came into contact with the immobilized immune complexes. Since 12 to 18 membranes were immunoblotted together (6 to 9 for each subcutaneous and intramuscular injection route), these membranes were transferred to 2 plastic boxes, each one containing the membranes subject to dilutions of the same serum. Since the light was stable for only a few minutes, it was important to work as quickly as possible. The membranes were positioned adjacent to one another in the box, in alphabetic or numeric order. Seeing that membrane A was subject to a 1:50 dilution of serum (for example) and membrane B subject to a 1:100 dilution (for example), the order of membranes was important so that a gradation of immune complex intensity could be visualized in the luminescence film.

The Amersham detection solutions 1 and 2 were allowed to come to room temperature and an aliquot of each was withdrawn using a Volac 100-1000 μl micropipette. The volume of each solution used for detection was dependant on the area of membrane being detected. The final volume required was 0.125 ml/cm^2 (as specified by Amersham), and for 9 membranes each having an area of 4 cm^2, the total volume of solution required was 36 cm^2 x 0.125 ml/cm^2 = 4.5 ml. Thus 2.25 ml of each detection solution was withdrawn

and transferred to individual clean 5 ml beakers.

Since it was important to work quickly during the chemiluminescent protocol, all other solutions and equipment were also prepared prior to detection. A piece of clingfilm (approximately 10 x 10 cm^2) was cut and positioned on a piece of paper towel, ensuring that the clingfilm was positioned flat on the towel without any folds or creases. The luminescence film which had been pre-cut was allowed to come to room temperature and the black plastic sleeve containing the film was transferred to a light sealed drawer for easy access during photographic development and printing. The amber bottles containing the 1+4 dilution of the developer and the 1+9 dilution of the fixer were also brought to room temperature. Approximately 200 ml of each was decanted into individual plastic boxes.

At this stage all solutions were prepared and the equipment positioned for easy access. The detection solutions (2.25 ml of each in individual beakers) were added together, swirled around and then poured over the membranes which had been positioned in the plastic box. It was important that the entire membrane surface was covered to ensure that all immune complexes came into contact with the solution. As soon as the solution was poured over the membranes, the timer was started and the membranes incubated for precisely 1 minute (as specified by Amersham). When the time had expired, a single membrane (e.g. membrane A) was removed from the plastic box using tweezers and excess detection solution drained off, by touching the underside of the membrane against the paper towel. The membrane was then positioned on the piece of pre-cut clingfilm. The transferral procedure was repeated for all membranes that were exposed to detection reagent and the membranes placed adjacent to one another on the clingfilm with their antigen/antibody sides facing upwards. The transferral of all membranes did not take longer than 60 to 90 seconds.

The clingfilm was then folded over the membranes themselves and the edges of this clingfilm pocket were sealed and any air bubbles and creases smoothed out. This latter step was important as any artefacts on the clingfilm (e.g. creases) were detected as interfering lines on the luminescence film. At this stage the chemiluminescent reaction had been generating light for 2 to 3 minutes, but because this light fell just within the visible spectrum (425 to 430 nm), it was not detected with the naked eye while the dark room lights were on.

The following section describes how this chemiluminescent light emission was captured on the luminescence film as well as the techniques used to develop and print the film, in order that the immune complexes be visualized.

5.2.2.3.2 Development and Use of a Working Protocol for Photographic Development and Printing of the Luminescence Film

The chemiluminescent protocol that was described in the previous section (5.2.2.3.1) was performed with dark room lights on. Because the protocol that is to be described makes use of a light sensitive luminescence film, the lights were switched off at this stage. However, it was permissible for a red safe light to be switched on throughout this protocol.

At this stage, the clingfilm pocket of membranes was transferred to the open Hypercassette. The pre-cut luminescence film was removed from the light sealed drawer, and a single 8 x 4 cm^2 strip was placed over the chemiluminescent light emitting membranes. Because the lights had just been switched off, it was often difficult to determine where the film should be positioned so as to cover the entire membrane surface. It was best then to wait a few seconds so as to become accustomed to the dark before

placing the film.

In the development stages of the protocol, it was not known how long the film should be exposed to the the light emitting membranes in order to quantitate light emission. Quantitation of light emission has been performed using photomultipliers[176] as well as luminometers measuring a photocurrent[184]. The only clues as to the length of exposure of the film to the developer was given by Thorpe et al.[185] who were the first researchers to monitor ECL immunoasays photographically. They suggested that the film only needed a 30 to 60 second exposure time to the membranes. However, Amersham alluded to an increase in the intensity of chemiluminescent light emission over approximately 10 minutes, after which the intensity began to decrease steadily (see Figure 5.6.).

If only a single exposure of the film to the membranes was to be made, then the remaining (stable) chemiluminescence would not be made use of and would thus be wasted. It was therefore decided to sequentially expose a series of films to the membranes. This technique would then confirm whether a particular immune complex incubated with a specific primary antibody was detectable on the film or not.

The film was initially exposed to the membranes for 3 minutes. This was performed by positioning the film over the membranes and then pressing down on the film with the knuckles to ensure maximum contact between the film and the membranes. The Hypercassette was then closed and the time noted. It was important not to move the cassette during exposure otherwise blurry photographs would have been printed. As soon as the first exposure period was complete, the Hypercassette was opened and the film removed using tweezers. A fresh piece of unexposed film was immediately placed over the membranes, the cassette closed and this second piece of film allowed to expose for 4 minutes. When

this exposure period was complete, the exposed film was removed and a third piece of unexposed film placed over the membranes and exposed for 5 minutes. The exposure periods were chosen as 3,4 and 5 minutes respectively in order that any decrease in chemiluminescent light intensity would be compensated for by an increase in exposure period. Hence the intensity of the immune complex visualized on the luminescence film should have been roughly the same for each film so as to allow for comparisons to be made between the blots on the 3 pieces of film.

As soon as the first piece of exposed film was removed from the Hypercassette using tweezers, it was immersed in the 1+4 dilution of the developer. Since these photographic chemicals were specific for developing X-ray film, the exposure periods of the film to the chemicals had to be adapted so as not to over- or underdevelop the film. Ilford suggested a 3 to 8 minute exposure period of the film to the developer. However, when this was tried the film became too dark which prevented detection of the immune complexes. Then a few runs of the entire protocol were performed using 1, 2 and 4 minute developing times. Although the shorter periods were an improvement, the film was still being subject to the developer for too long.

As an alternative technique, it was decided not to set a time for exposure of the film to the developer. The film was thus immersed in the developer solution using tweezers, and the box containing the developer and film was positioned close to the red safe light. For the more concentrated UV-irradiated DNA used as antigen, the film was removed from the developer when black immune complexes were seen on the film while it was in the developer. For the less concentrated UV-irradiated DNA (less than 0.01 mg/ml DNA) sample used as the antigen, the film was removed when it had become sufficiently "grey". Usually, it took between 15 and 30 seconds for this to occur.

As soon as the film was removed from the developer using tweezers, it was immersed in the 1+9 dilution of the fixer. Ilford suggested a 3 to 5 minute exposure period, but to speed up the protocol, 1,2 and 4 minute exposure periods were experimented with. Because a 1 minute exposure period was not visually different from a 4 minute exposure time, this former time was chosen and used in all subsequent work.

Once the film had been fixed, it was removed from the fixer using tweezers and washed under a gentle stream of water to remove excess developer and fixer. The film was dried using a hairdryer set at the lowest heat setting.

There was sufficient time for the first piece of exposed film to be developed and printed before the second piece was removed from the Hypercassette and developed and printed. This second piece was then immersed in the developer for 15 to 30 seconds and transferred to the fixer where it was immersed for 1 minute. It was washed under a stream of water and allowed to dry before the entire developing and printing procedure was repeated for the third piece of film. If a second series of membranes were immunoblotted at the same time, then the entire chemiluminescent protocol as explained in Section 5.2.2.3.1 and above, was repeated.

This entire protocol usually took no more than 15 minutes to perform. If this chemiluminescent technique was being used on a regular basis in a clinical or analytical laboratory, it provides a means of analysis of several samples over a short space of time.

Only when the developing and printing protocol was complete, and when the pre-cut unexposed film had been returned to its drawer, could the lights be switched on and the film analysed and interpreted.

However, before the chemiluminescent protocol was performed, it was important to ensure that all the chemicals were working and not past their expiry date. The detection system was checked by pipetting 0.5 ml of each detection solution into a 5 ml beaker. With the lights switched off, 3 μl of HRP-antibody conjugate was added to the detection solution using a Gilson 2-10 μl micropipette. The solution was swirled around and blue light seen. In most cases the blue light could be seen for 10 minutes or longer, but this was not an indication of the stability of the light in this work, because diluted, and not stock HRP-antibody conjugate was used in the protocol described in this work. It merely confirmed that the chemiluminescent protocol was working. This procedure was performed whenever immune complexes could not be visualized on the luminescence film. The detection system did, however, produce blue light whenever it was tested and was stable for at least 12 months if stored in the refrigerator.

The luminescence film was also checked to see that it yielded a clear background when developed and printed. A pre-cut piece of film was immersed in the developer and fixer for the required periods of time before being washed and dried. The background was clear when tested during the first 4 to 6 months of use. After that time, the film became too dark when developed and printed and was thus discarded and a new pack of film was bought. The developer also had a limited shelf life. When new film was bought, the developer was also discarded and bought fresh.

In order to develop a rapid means of quantitation of the emitted light, an attempt was made to use a Macam Spectroradiometer (SR 9010-PC) attached to a Samsung Personal Computer. This instrument allows emitted light (UV, visible and chemiluminescent) to be captured using a diffuser and light guide, and then electronically converted to a light output reading by means of a monochromator. The

chemiluminescent protocol was performed on a series of immunoblotted membranes, and once the detection solution was added to the membranes, the probe was positioned over a light emitting membrane. No reading was however registered by the radiometer when the spectrum was scanned from 400 to 450 nm over a 15 minute time period. Seeing that it was very difficult to detect the emitted light in the dark, using the naked eye, it was not surprising that the emissions were not detected using the radiometer. If this technique had been successful, the emitted light could have been quantitated and correlated to light emissions from known thymine dimer concentrations when the chemiluminescent protocol was performed. In this way, thymine dimers generated at these known low DNA concentrations could have been calculated. Since the light could not be detected, this technique was not developed any further.

As will be explained in Section 6.3.2, the luminescent films containing immune complexes of varying intensities, were also quantitated using a GS 300 Transmittance/ Reflectance Scanning Densitometer attached to a Varian 4270 Chart Recorder. This instrument allowed the density of the immune complex on the luminescence film to be determined against the individual background reading for that film. This technique was used for quantitating those complexes at or near the cut-off dilutions of the antibody for a specific DNA concentration. These results will be discussed in Section 6.3.2.

5.2.2.4 Summary of the Chemiluminescence Protocol for Thymine Dimer Quantitation in UV-Irradiated DNA

1. The detection solutions were brought to room temperature and equal volumes pipetted into individual beakers to give a final volume of 0.125 ml/cm^2 of membrane.

2. The membranes were drained of excess PBS buffer and placed in a fresh container. The detection reagent was added directly to the antigen/antibody surface of the membranes.
3. The membranes were incubated for exactly 1 minute.
4. Excess detection reagent was drained off and the membranes wrapped in clingfilm. Any creases or air pockets were smoothed out.
5. The membranes were placed (antigen/antibody side up) in the Hypercassette. It was important to work as quickly as possible, minimizing the delay between incubation with detection reagent and exposure to the film.
6. The lights were switched off and a sheet of pre-cut luminescence film was placed over the light emitting membranes. The cassette was closed and the film exposed for 3 minutes.
7. The film was removed and a fresh piece of film positioned over the membranes for 4 minutes.
8. While the second piece of film was being exposed, the first piece was immersed in developer for 15 to 30 seconds, and then fixer for 1 minute.
9. This luminescence film was washed under a gentle stream of water and dried using a hairdryer.
10. The second, and any subsequent pieces of exposed film, were also immersed in developer and fixer.
11. The films were analysed for the presence of immune complexes at specific antibody dilutions.

Chapter 5 has thus described the development and use of a working immunoblotting protocol with chemiluminescent detection for the quantitation of cis-syn thymine dimer in UV-irradiated DNA. In Chapter 6, attention is now turned to results and discussion of the purification techniques used in this work (ammonium sulphate precipitation of antibody and affinity chromatography), as well as the abovementioned

immunoblotting protocol with ECL detection and quantitation. As an extension of this work, DNA of 0.025 mg/ml was irradiated and analysed by immunoblotting with ECL. The cut-off antibody dilutions were correlated to thymine dimer yields (using a calibration graph). These results were then used to support a mechanism as proposed by Thomas[54] for the acetophenone photosensitized dimerization of thymine in DNA. Here, a comparison was made between experimental (determined by immunoblotting) and simulated dimer yields (outputted by the computer programme, CAKE).

CHAPTER 6 RESULTS AND DISCUSSION

The main aim of the work reported in this thesis was the development of an immunoassay technique for the quantitation of thymine dimer in UV-irradiated DNA. This would be a more sensitive technique for thymine dimer quantitation than the HPLC techniques currently being used in these laboratories and would allow the extension of thymine dimer quantitation from *in vitro* (free thymine or calf thymus DNA) aqueous systems to quantitation in *in vivo* cellular systems. The experimental techniques employed in the development of an immunoassay protocol were described in Chapters 4 and 5. Briefly, DNA-acetophenone-PBS solutions of varying concentrations were prepared and irradiated with ultraviolet light of wavelengths greater than 290 nm to cause photosensitized production of the cis-syn thymine dimer. Injection of this UV-irradiated DNA antigen into laboratory rabbits allowed antibodies against the thymine dimer to be generated by the rabbits' immune system. The antibodies were collected by bleeding the ear vein of the rabbits. Purification of the crude serum was performed by the classical ammonium sulphate protein precipitation technique as well as by the more sophisticated technique of affinity chromatography. The immunoassay technique of immunoblotting was developed using crude serum (containing thymine dimer antibodies) and quantitation of the thymine dimer was performed using enhanced chemiluminescence detection of the antigen/antibody complexes.

This chapter deals with an evaluation of the purification techniques employed in this work, as well as presenting results obtained from the immunoblotting analysis of crude serum samples. Included here will be a discussion of the sensitivity of this immunoblotting technique in comparison with the HPLC system currently being used. Thereafter the

applicability of immunoblotting for quantitating thymine dimer lesions at DNA concentrations undetectable by HPLC will be discussed. To this end, the construction of a calibration graph (cut-off antibody dilutions versus thymine dimer concentrations) will be discussed. Additionally the mechanism of acetophenone photosensitized dimerization of thymine as proposed by Thomas[54] for 0.1 mg/ml to 1.0 mg/ml DNA samples was tested at 0.025 mg/ml DNA by irradiating DNA and analysing the dimer yields by immunoblotting. Support for the proposed mechanism at this low (0.025 mg/ml) DNA concentration was made by comparing simulated (obtained by using the computer programme, CAKE) and experimental (determined by immunoblotting) data.

6.1 PURIFICATION OF THE CRUDE SERUM

In this work two techniques (ammonium sulphate precipitation and affinity chromatography) were employed for the purification of proteinaceous antibodies. These UV-DNA antibodies were generated by rabbit immunocompetent cells and secreted into the rabbit blood stream. Blood was obtained from the marginal ear vein of the rabbit, and the serum (containing the antibodies) separated from the red blood cells by centrifugation. Aliquots of this crude serum were frozen in Eppendorf vials to prolong their lifespan and prevent microbial degradation. The vials were thawed whenever the serum was required for immunoblotting analysis or purification.

Protein purification is intrinsically a delicate procedure to undertake because of the fragile conformations of proteins which are crucial in controlling cellular metabolism. The degree of purification and percentage recovery required for a specific protein dictates the technique(s) which is to be used. Precipitation methods employing ammonium and sodium sulphate, ethanol, dioxane and polyethylene glycol for purification are inherently

crude, but do provide a starting point for gross purification. Chromatographic methods making use of sophisticated technology and instrumentation provide a more selective and expensive means of separating proteins from each other. In this research, both precipitation and chromatography were employed in purifying crude serum containing thymine dimer antibodies.

6.1.1 A COMPARISON OF AMMONIUM SULPHATE PRECIPITATION AND AFFINITY CHROMATOGRAPHY AS PURIFICATION TECHNIQUES

The purification of proteins by precipitation required that a saturated solution of ammonium sulphate be prepared. Ammonia solution was used to adjust the pH of the ammonium sulphate solution to 6.5. Crude serum was stirred using a magnetic stirrer and the saturated ammonium sulphate supernatant added to reach 50% saturation at which antibody precipitation occurs. The solution was adjusted to pH 7.8 (0.5 M NaOH), after which it was stirred on a magnetic stirrer for 2 to 3 hours while the protein precipitated. Centrifugation (2 x 15 minutes, 12000 rpm) separated the protein from the supernatant which contained serum proteins, amongst other biomolecules. The protein was re-suspended in PBS buffer and the entire procedure repeated to remove extraneous material from the precipitated fraction. The purified components were finally dialysed against large volumes of 50 mM phosphate buffer to remove excesses of ammonium sulphate salt.

In essence, this technique provides a satisfactory, yet gross means of purifying all antibody classes (IgG, IgM, IgA and IgE) from contaminating serum constituents and has been used for such purposes since it was initially devised by Kendall[186] for protein (gamma globulin) purification in 1937. Phillips and Kalthoff[135], who prepared monoclonal antibodies for specific binding to pyrimidine dimers in

RNA, precipitated the antibodies from ascites fluid with 50% saturated ammonium sulphate. This technique was also applied by Eggset et al.[187] prior to affinity absorbing UV-DNA antibodies onto a DNA-Sepharose column.

However, the drawbacks of ammonium sulphate precipitation in the work presented here lie not in the application of the technique, but in the small volumes of serum (500 μl or less) available for purification after a bleed of the rabbit. Although much of the developmental work for this protocol used the i/v T serum (approximately 50 ml available in total for purification and immunoblotting work), antibody purification using such small volumes of serum is a delicate procedure which cannot be accomplished with a high degree of success if quantitative work is required.

A further drawback of this technique lies in the fact that a pH of 7.8 must be established for protein precipitation of immunoglobulins, non-immune serum globulins and albumin to occur. However, alternative methods[140] suggest a pH of 7.4 be established in order for precipitation to occur. Although this pH difference is only 0.4, the fact that there is a difference suggests the range of proteins (whose pI falls between 7.4 and 7.8) which will precipitate using this ammonium sulphate technique. Thus if the purification of a single antibody class (e.g. IgG) from other antibody classes or contaminating serum proteins is required, then ammonium sulphate precipitation does provide a drawback in terms of its resolving powers. However, if crude purification is all that is required (i.e. ammonium sulphate precipitation of antibodies prior to chromatographic separation), then ammonium sulphate precipitation does initially serve an important function.

As an alternative to the crude purification technique

discussed above, a chromatographic technique for antibody purification was developed to separate immunoglobulins from contaminating serum components. The techniques of ion-exchange chromatography, hydroxylapatite chromatography and hydrophobic interaction chromatography have all been used elsewhere[142] in immunological applications for purification of antibodies. However, these separations (relying on differences in charge, shape and hydrophobic character of the antibody molecules) are extremely specific, usually producing highly pure antibodies for use as antigens or ligands in analytical or clinical applications. Such applications were not required in this work and hence the technique of affinity chromatography was employed to demonstrate chromatographic separation of immunoglobulins.

Affinity chromatography relies on the specific interaction of proteins or glycoproteins with ligands which are immobilized on an insoluble matrix. For the application at hand, Protein A (a cell wall protein expressed by the bacterial species *Staphylococcus aureus*) interacted with the antibody molecules. Extensive work by Swedish researchers in the 1970's elucidated that only the IgG antibody molecule (comprising 75 to 80% of the total antibody population) interacts with Protein A, hence effecting separation of this antibody class from other antibodies (IgM, IgA and IgE) and serum proteins.

An affinity chromatography cartridge (containing the Protein A ligand immobilized onto a cellulose matrix) was attached to a Waters Delta Prep 4000 preparative HPLC system with a Waters Tunable 486 UV absorbance detector and Waters 745 Data Module. Seeing that neither the preparative HPLC system or the affinity cartridge had been used in these laboratories, development of a protocol for antibody purification went hand-in-hand with becoming familiar with the workings of the HPLC.

A 0.05 M Tris/HCl, 0.1 M NaCl, pH 8 buffer solution was used to load a diluted serum sample onto the Memsep 1000 affinity chromatography cartridge. Thereafter a 0.1 M glycine/HCl solution,pH 2.5 desorbed the IgG component from the cartridge, the fraction was collected and confirmed to be IgG.

Results of the chromatographic separation of IgG using affinity chromatography were illustrated in Sections 4.2.3.8.2 to 4.2.3.8.4, where optimum separation was achieved using a 100 μl rheodyne loop volume and the following gradients generated by the HPLC solvent delivery system:
Tris/HCl pH 8.0 - 1.5 ml/min - 0 to 2 minutes and
glycine/HCl pH 2.5 - 1.5 ml/min - 2 to 20 minutes.
The results obtained on injection of commercial rabbit IgG onto the Memsep cartridge were discussed in Section 4.2.3.9.

In immunological applications, the percentage recovery of antibody using a particular purification technique is most easily carried out by radiolabelling the antibody, purifying the serum sample and performing scintillation counting on the resultant fraction. Seeing that radioimmunoassays were not utilized in this work an alternative technique was established. A solution of commercial rabbit IgG (0.96 mg/ml in PBS) was prepared and injected onto the preparative HPLC. The maximum absorbance of the IgG fraction on the Waters 486 Tunable absorbance detector was used to convert the experimentally determined absorbance to a concentration reading. The Beer-Lambert Law, $A = \epsilon Cl$ (with $\epsilon = 1.43$ $(mg/ml)^{-1}$ cm^{-1} as determined in Section 4.2.3.1 and $l = 1$ cm) gave a concentration of 0.909 mg/ml for the sample which had passed through the Memsep cartridge. This corresponded to a 94% recovery of the commercial rabbit IgG. If an extinction coefficient of 1.50 $(mg/ml)^{-1}$ cm^{-1} is used[135], then the recovery is 90%

efficient.

In this work it was not necessary or relevant to quantitate absolute antibody production in the various serum samples obtained after bleeding the rabbits. Seeing that factors beyond the control of the researcher (genetic predisposition of the animal to the immunogen, age and immunological status of the animal) influence antibody production, quantitation of these serum samples would have served no purpose in this work. As will be shown in Section 6.3, correlation of cut-off antibody dilutions (obtained by immunoblotting) to thymine dimer concentrations was made without knowing or calculating the specific thymine dimer antibody concentrations in any serum samples.

In an article entitled "Liquid Chromatography of Immunoglobulins", Regnier[142] comments that the majority of antibody purification regimes employ multi-step processes using one or more separation techniques to bring about efficient purification and/or separation. In this context, ammonium sulphate precipitation is usually followed by or incorporated with ion-exchange chromatography to produce antibodies which contain less than 10% protein by mass. Another example is given of an antibody preparation that was pre-fractionated onto a Protein A column and was resolved into its three idiotypic components using a hydroxylapatite column[148]. It seemed pertinent to apply this logic to the purification techniques that were used individually in these laboratories, i.e. to follow protein precipitation with HPLC. An i/v T serum sample was subject to a single ammonium sulphate precipitation, the protein re-suspended in PBS buffer and a diluted sample thereof injected onto the Memsep cartridge. Although quantitation of antibodies was not important in this work, areas integrated under the peaks by the Waters 745 Data Module give an indication as to the percentage IgG in any particular serum sample. Depending on the sample used, the

IgG consisted of approximately 30% of the crude sample (i.e. without ammonium sulphate precipitation) injected onto the Memsep cartridge. When the ammonium sulphate precipitated antibody was passed through the Memsep, the area percentage increased to approximately 95%. If a 4 fold purification of the antibody using ammonium sulphate precipitation is assumed[138], then coupling these two techniques results in an approximate 12 fold purification of the IgG component. This figure is in the range of that given for DEAE ion-exchange chromatography, where the antibody classes and subclasses may be separated from one another. (Mahler and Cordes[138] note that crystallization of a protein results in a 250 fold purification - far superior to the purifications achieved using chromatography, but much more difficult to perform.) As was noted above, affinity chromatographic purification of proteins yielded a percentage recovery of greater than 90%. If maximum recovery of an antibody class from a particular serum sample is important for the research at hand, then affinity chromatography is superior to the 80% recovery of antibodies that can be achieved using ammonium sulphate precipitation[138].

In contrast to the high recoveries and successful applications of the affinity chromatography techniques mentioned above, a few features of affinity chromatography must be discussed in terms of the disadvantages they might provide in purification regimes. Included here is the inability of this technique to separate antibody subclasses from one another, changes in antibody conformation during desorption with the glycine/HCl buffer and the removal of nucleases using this technique. These are discussed in the following Section (6.1.2).

6.1.2 LIMITATIONS OF AFFINITY CHROMATOGRAPHY

Section 4.2.3.8 discussed the development and use of an

affinity chromatography protocol to separate the IgG antibody component from contaminating serum proteins and other immunoglobulin classes. The technique was chosen from a range of chromatographic applications since it demonstrated the purification of antibody molecules from whole serum without separating the immunoglobulin classes from one another. At the same time the technique allowed separation of the various serum proteins from the antibody of interest (IgG) and eluted the IgA, IgM and IgE antibodies with the serum proteins. Although the technique proved successful in this work because a high degree of resolution was not required, affinity chromatography does have drawbacks. These will now be highlighted and discussed sequentially.

As was stated above, in the work presented here it was not necessary to separate the antibody classes from one another. But if resolution of these classes is integral to the research being undertaken, then an alternative technique to affinity chromatography must be chosen to effect separation of the antibody classes from one another.

A range of techniques are currently available to serve this purpose. Separation can be achieved using electrophoresis where the differences in size and charge of the various antibody classes are used to bring about resolution. Nussenweig et al.[188] fractionated mouse antiserum by zone electrophoresis but achieved only partial separation of IgG_1 and IgG_2 antibodies because of the small diferences in electrophoretic mobility of these two types of antibody. However, electrophoresis has in many instances now been replaced by ion-exchange chromatography as a separation technique. Anion exchange chromatography using a Waters PROTEIN-PAK DEAE-5PW column was used successfully for the separation of IgG, IgM, IgA, transferrin and albumin[157] (hence separation of the antibody from the contaminating serum proteins was achieved). This operation requires the

careful simultaneous control of ionic strength and pH gradients using Tris and NaCl buffers at alkaline pHs (7 to 8.5) to bring about efficient separation. This technique has been used to purify monoclonal antibodies of a variety of idiotypes from both ascites and tissue culture fluid. Ion-exchange chromatography also provides a means of resolving immunoglobulin sub-classes, as was demonstrated for the separation of mouse IgG_3 and IgG_{2b} from IgG_1 and IgG_{2a}[145].

The abovementioned techniques exploit subtle differences in size, charge and electrophoretic mobility of the antibody molecules, and hence effect differing degrees of resolution of the antibody population according to the specific technique. Hence, prior to the researcher chosing a method for chromatographic separation of antibodies, it is important to establish the degree of purity required. If the application demands resolution of the various crude antibody components on an idiotypic basis, then ion-exchange chromatography or the more sophisticated techniques of hydroxylapatite or hydrophobic interaction chromatography must be used. If, however, the application demands only the separation of antibody from contaminating serum protein, then affinity chromatography provides a cheap option which can find use in both analytical or semi-preparative operations depending on the size of the Memsep cartridge chosen and the flow rates of the buffers used.

If the IgG fraction is to be collected in a semi-preparative or preparative application and used for structural analysis of the IgG, then affinity chromatography is deleterious in that it brings about structural and conformational changes to the antibody molecule: as was outlined in Section 4.2.3.7.1, a 0.05 M Tris/HCl, 0.1 M NaCl, pH 8 buffer solution was used to load the IgG onto the Memsep cartridge, while 0.1 M glycine/HCl, pH 2.5 interfered with the binding of the IgG/Protein A

complex and thereby released the IgG for detection. It is the acid environment (pH 2.5) which induces changes to the IgG, and these are manifest as changes to the three dimensional structure of the antibody. In particular, a general expansion of the molecule is brought about due to an increase in the angle between the two F_{ab} arms[189].

Another drawback as to the applicability of affinity chromatography is given by Munns and Liszewski[190] who note that the technique does not always eliminate nuclease activity from the antibody preparation. The removal of nucleases is important in preventing degradation of the material under investigation. It is therefore recommended that additional steps be taken (in conjunction with affinity chromatography) to ensure nuclease removal or inactivation. This may be accomplished by combining ion-exchange and affinity chromatography techniques although a 20 to 40% loss of antibody population usually accompanies nuclease removal. Alternative procedures which may be employed include the use of charcoal and alum for nuclease removal.

This section (6.1) has discussed the techniques of ammonium sulphate precipitation and affinity chromatography in terms of their applications and limitations in the purification of antibodies. Apart from demonstrating that purification of antibodies can be achieved using the Memsep cartridge and Delta Prep 4000 HPLC (both of which were newly acquired in this laboratory), it had been thought that pure antibody preparations were necessary in order to carry out the immunoblotting protocol. To this end, Section 6.2. discusses aspects of the immunoblotting with ECL detection chosen to provide a more sensitive means of thymine dimer quantitation than HPLC currently used in these laboratories.

6.2 IMMUNOBLOTTING ANALYSIS OF IRRADIATED DNA WITH ENHANCED CHEMILUMINESCENT QUANTITATION OF ANTIGEN/ANTIBODY COMPLEXES

The research presented here outlines the development of an immunoassay protocol for the *in vitro* detection of thymine dimers in UV-irradiated DNA. The technique of immunoblotting was chosen over the radioimmunoassay and immunofluorescence immunoassays because of the ability of immunoblotting to reveal lesions induced by low fluences of UV radiation (< 10 J/m^2). Immunoblotting also has the advantage of requiring unsophisticated instrumentation in comparison with that required by radioimmunoassay and immunofluorescence.

An adaption of the immunoblotting protocol presented here was first described by E.M. Southern[83] in 1975 to allow for proteins and nucleic acids (e.g. UV-irradiated DNA) to be immobilized on a membrane matrix. In this work, subsequent processing of the immobilized UV-irradiated DNA antigen involved blocking unreacted areas of the membrane surface with 2% dried milk in PBS-Tween solution, followed by incubation of the membrane with diluted serum samples (containing antibodies generated against thymine dimers of the UV-irradiated DNA). The membrane was finally incubated with a second antibody (specific for the primary antibody) bound to the enzyme horseradish peroxidase. At the end of the 24 hour protocol, each immobilized blot consisted of antigen, primary antibody and secondary antibody conjugated to the enzyme and bound together as a complex by a series of specific antigen/antibody interactions.

In this work the technique of enhanced chemiluminescence was chosen to quantitate thymine lesions generated in the UV-irradiated DNA. Chemiluminescence has the advantages of cheap and stable reagents, rapid assay times and superior sensitivities over ELISAs and radioimmunoassays that could

have been chosen as quantitation techniques. Enhanced sensitivity of the assay is gained by the addition of chemical enhancers to the chemiluminescent detection solutions. These enhancers provide a 80 to 100 fold enhancement of sensitivity[184].

In the protocol employed here, the antigen/antibody complexes immobilized on the Immobilon-N membrane were overlaid with ECL detection solutions (luminol-H_2O_2-enhancer) which permitted the peroxidase enzyme to catalyse the production of light (425 to 430 nm) generated during the course of conversion of luminol to the aminophthalate dianion. The light was captured on luminescence film which was developed and printed using standard photographic chemicals. The emitted light intensity was correlated to antigen bound to the membrane surface and hence allowed thymine dimer quantitation of the immobilized UV-irradiated DNA.

This section (6.2) describes the results of the immunoblotting protocol that were obtained during the development stages of the protocol. Then the discussion will consider antibody production in terms of the injection routes chosen for the immunization of rabbits. Thereafter the applicability of both immunoblotting and enhanced chemiluminescence will be discussed from the perspective of the problems experienced during the running of the protocols.

6.2.1 RESULTS OF IMMUNOBLOTTING ANALYSIS TO DETERMINE OPTIMUM CONDITIONS UNDER WHICH TO BLEED THE RABBIT

Immunizations of the UV-DNA antigens were performed once a week over a four week period (see Section 4.2.2.3). Rabbits were bled prior to injection as well as 10 and 20 days after the final injection to obtain the serum containing

the thymine dimer antibodies. The immunoblotting protocol was performed on each sample of serum obtained from a single rabbit bleed. Seeing that two rabbits received the same immunogen via different injection routes (intravenous, intramuscular and subcutaneous), one immunization schedule (4 injections and 6 bleeds per rabbit) necessitated the performance of 12 individual protocols with ECL detection in order to determine the optimum conditions under which the rabbits should be bled to obtain maximum antibody titres.

Quantitation was performed by examining the cut-off antibody dilution of a particular serum sample, i.e. that antibody dilution where the immune complex was no longer visible on the luminescence film. Results are presented as cut-off antibody dilutions (y-axis) versus days of immunization/bleeding (x-axis). The performance of these protocols not only served to develop the techniques of immunoblotting and ECL but also allowed one to determine when the rabbit should be bled so as to obtain maximum antibody concentration. The higher the antibody titre, the more antibody available.

Results are presented in tabular form in Tables 6.1., 6.2. and 6.3. while they are represented graphically in Figures 6.1., 6.2. and 6.3. Figure 6.4. illustrates a typical luminescence film containing antigen/antibody complexes viewed after development and printing of the films.

Days	Subcutaneous Cut-off dilution	Intravenous Cut-off dilution
0	0	0
7	1:100	1:200
14	1:1200	1:3600
21	1:3600	
24		1:16200*
31	1:5400	

* A terminal bleed was required at 24 days because of the problems which arose with the intravenous injection route of the UV-DNA antigen (see Section 4.2.2.3).

Table 6.1. Cut-off antibody dilutions of the thymine dimer containing serum generated in response to the subcutaneous and intravenous injection of 1.0 mg/ml DNA.

Days	Subcutaneous Cut-off dilution	Intramuscular Cut-off dilution
0	1:400	0
7	1:800	1:800
14	1:1200	1:1200
21	1:3600	1:3600 or 1:5400*
31	1:7200	1:5400
41	1:8000	1:9000

* Hybond versus Immobilon-N membranes (see Section 6.2.3.).

Table 6.2. Cut-off antibody dilutions of the thymine dimer containing serum generated in response to the subcutaneous and intramuscular injection of 1.0 mg/ml DNA.

Days	Subcutaneous Cut-off dilution	Intramuscular Cut-off dilution
0	1:800	1:400
7	1:1200	1:800
14	1:3600	1:3600
21	1:5400	1:5400
31	1:7200	1:8000
41	1:7200	1:9000

Table 6.3. Cut-off antibody dilutions of the thymine dimer containing serum generated in response to the subcutaneous and intramuscular injection of 0.5 mg/ml DNA.

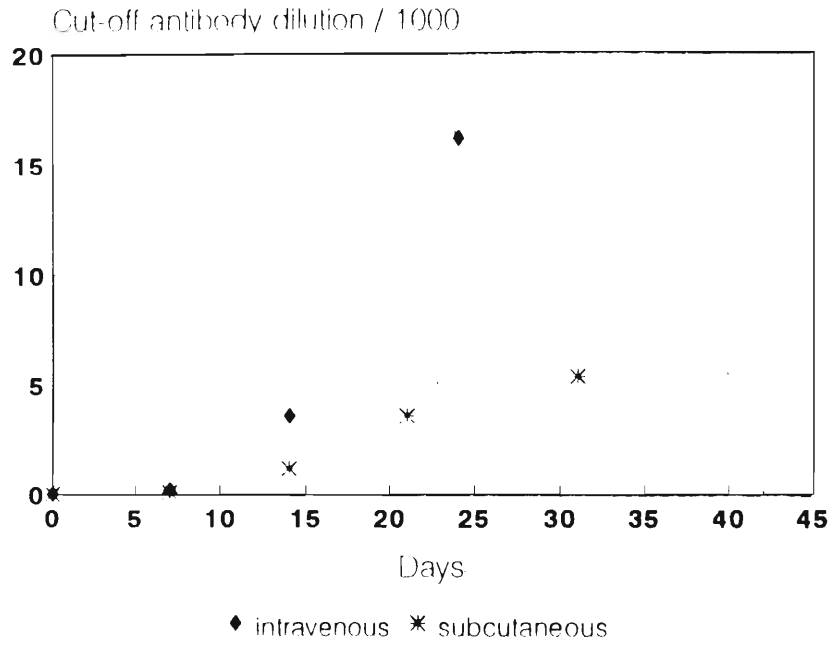


Figure 6.1. Cut-off antibody dilutions of the thymine containing serum generated in response to the subcutaneous and intravenous injection of 1.0 mg/ml DNA.

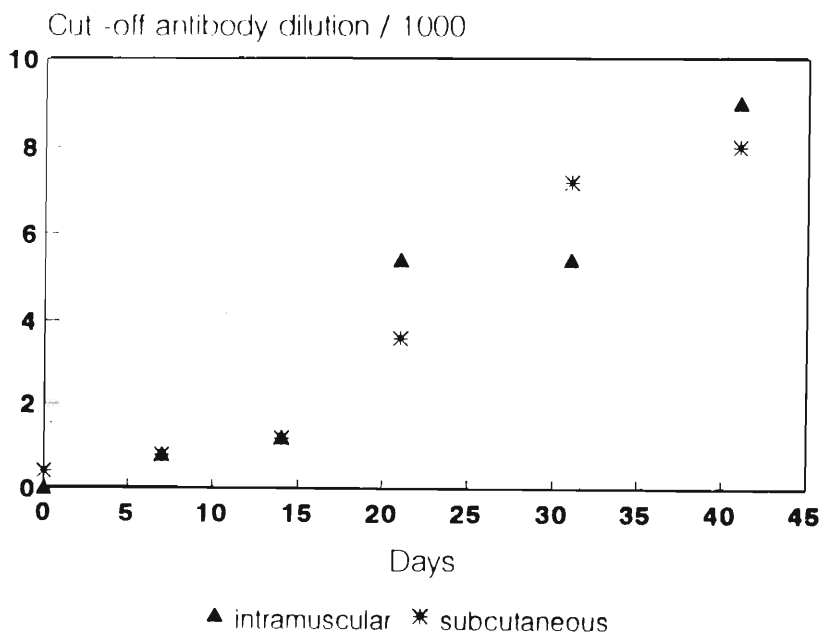


Figure 6.2. Cut-off antibody dilutions of the thymine dimer containing serum generated in response to the subcutaneous and intramuscular injection of 1.0 mg/ml DNA.

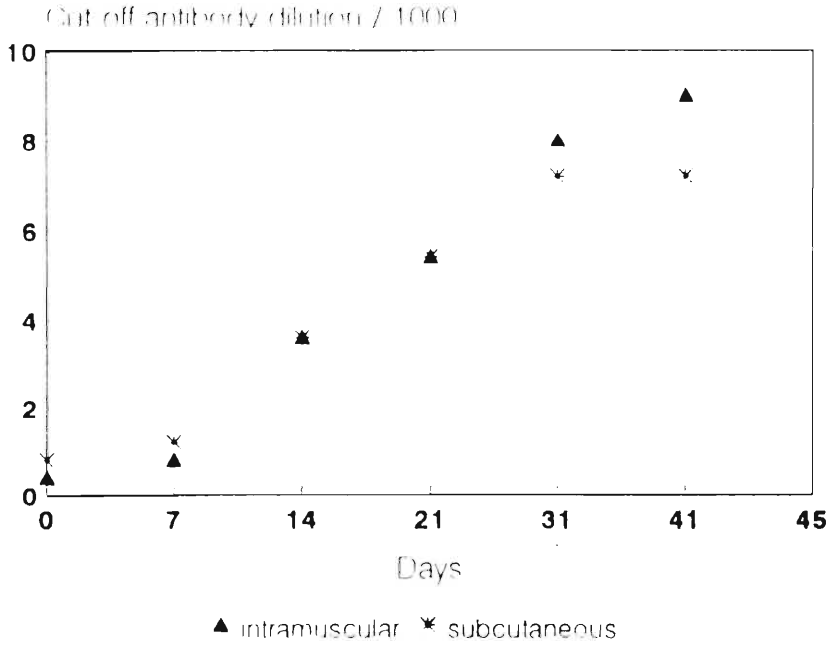


Figure 6.3. Cut-off antibody dilutions of the thymine dimer containing serum generated in response to the subcutaneous and intramuscular injection of 0.5 mg/ml DNA.

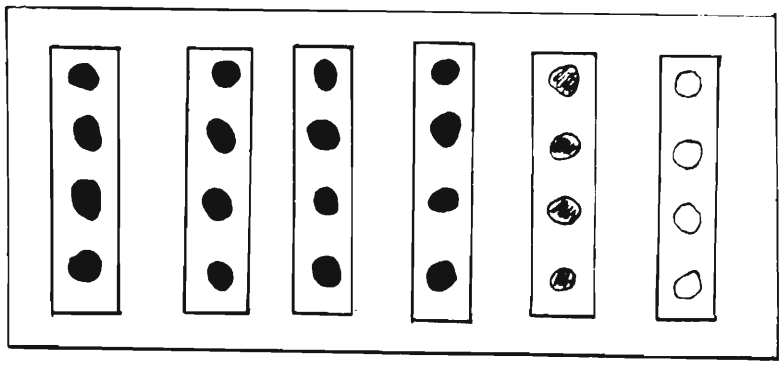


Figure 6.4. Typical luminescence film illustrating a gradation in antigen/antibody blot intensity in relation to increasing serum dilutions.

Because antibody production is a biological process over which little control can be exercised by the researcher, it is not possible to draw conclusions as to the extent of antibody production in response to the UV-irradiated DNA. The higher antibody titre in a particular serum sample, the more it can be diluted for use in the immunoblotting protocol, and the higher the cut-off dilution which is achieved in the assay. In the results presented here, the highest antibody titre at day 41 implies that serum taken around this time is optimum for analysis. It must be noted, however, that the same 'subcutaneous' rabbit received immunizations of 1.0, 1.0 and 0.5 mg/ml UV-DNA in three succesive immunization schedules. In the same manner, the 'intramuscular' rabbit received doses of 1.0 and 0.5 mg/ml UV-DNA in a series of two immunizations. Hence the 1:800 dilution of serum for the second immunization schedule is specific only for that serum. Thus a 1:800 dilution of serum in the third immunization schedule cannot be correlated to the former 1:800 dilution because the assay does not provide a means of determining absolute antibody concentrations in the various sera samples.

From an immunological point of view, it is advantageous for bleeds to be performed months after immunization of the antigen, in order to monitor the long term course of acquired immunity of the animal. However, such a procedure served no purpose in this work and because only three rabbits were available for immunization, it was important to begin subsequent immunizations only weeks after the final bleed of the previous schedule was complete.

6.2.2 EFFECT OF INJECTION ROUTE ON ANTIBODY PRODUCTION

Although few conclusions can be inferred as to the extent of antibody production during the course of immunizations, it is generally believed that the efficiency of stimulation of the immune response is related to the site of

innoculation[131]. In this work, three injection routes (intravenous, intramuscular and subcutaneous) were used, with success shown in the latter two cases. The main reasons for the differences in efficiency (quoted as intravenous < intramuscular < subcutaneous by Hurn and Chantler[131]) are the speed with which the antigen is lost from the site of injection and the likelihood of it passing through the lymph nodes or other centres of immunologic activity. However, these factors are extensively affected by the use of oily adjuvants which can stimulate a local cellular reaction thereby releasing antigen over a period of several weeks or even months.

Because Freunds Incomplete Adjuvant was used to stimulate antibody production in rabbits in this work, the subcutaneous and intramuscular injection routes were chosen so as not to cause loss of condition of the rabbits. Hurn and Chantler[131] note that subcutaneous or intradermal injection invariably leads to ulceration, but provided the sites are well chosen no problems are experienced. Injection of the immunogen by sucbcutaneous and intramuscular means in this work was compatible with the rabbits and no problems were experienced during the course of either the three or two immunization schedules. Problems related primarily to the generation of the condition of fatal fat embolism (see Section 4.2.2.3) were experienced with injection of Freunds Adjuvant using intravenous injection.

The following sections (6.2.3 and 6.2.4) highlight aspects of the immunoblotting and ECL assays that were considered in this work in order to provide a viable means of identifying and quantitating thymine dimer lesions in UV-irradiated DNA.

6.2.3 APPLICABILITY OF IMMUNOBLOTTING

Immunoblotting has been used successfully in this work to detect thymine dimers in UV-irradiated DNA. In addition, immunoblotting allowed the quantitation of thymine dimers at dimer concentrations undetectable by HPLC (see Section 6.4) and thus provides a more sensitive means of dimer detection than HPLC. Immunoblotting also has the advantage of not requiring pre-treatment of the DNA antigen prior to analysis. Furthermore, a single 400 μl irradiated aliquot of DNA allowed several more analyses to be performed using immunoblotting, as only 5 μl of sample was required for immunoassay analysis in comparison with the 30 μl of hydrolysate injected onto the Ultracarb 5 ODS 30 column during HPLC analysis.

The work presented in this thesis discusses the development of an immunoblotting protocol for thymine dimer quantitation. The technique is however only as good as the chemicals and reagents used in its application. To illustrate this point, an immunoblotting protocol with ECL detection was performed using two different membranes for immobilization of the antigen/antibody complexes. In the first case, the charge modified Immobilon-N membrane (chosen for this work) was used. A nylon based Hybond membrane was also used in the same assay with all other chemicals and reagents the same. Results indicate that a 1:5400 dilution of serum (obtained after immunization of a 1 mg/ml DNA antigen injected intramuscularly) was not detectable using Hybond, but with the Immobilon-N membrane the 1:5400 dilution was clearly visible. An observation such as this is indicative of the extent to which the sensitivity of the assay is dependant on the reagents and equipment used during experimentation.

Thus, in the work presented here, reagent concentrations and incubation times were optimized to provide the

strongest signal of the immune complex against the clearest background (Section 5.2.1.7). One factor which could not be controlled with a large degree of success was the way in which the membrane itself dried while the blotted UV-DNA was drying. This protocol is discussed in Section 5.2.1.7.2. As was noted, the membranes were pre-wet in methanol, water and PBS prior to transferral to an adapted ELISA plate positioned over an open plastic box. Each membrane was shaken by hand to remove excess solvent prior to being blotted and it was found that the membrane dried erratically in comparison with the rate at which the UV-DNA dried on its surface. In a few cases the membrane became very white prior to the antigen drying (indicative of the membrane drying out). Although the situation was much improved by moving a stream of air across the membrane surface there was no absolute control over the rate of drying, especially for the more concentrated and viscous DNA samples which took longer to dry. The best way of overcoming the problem was to move a stream of air across the membranes and transfer a membrane to the dried milk solution as soon as the antigen blots were dry. As will be outlined in Section 6.2.4, this feature did affect visualization of the antigen/antibody complex on the luminescence film.

6.2.4 APPLICABILITY OF ENHANCED CHEMILUMINESCENT QUANTITATION OF IMMUNE COMPLEXES

ECL was used in this work to detect and quantitate antigen/antibody complexes immobilized on a membrane surface and thus together with immunoblotting, these techniques allowed the successful quantitation of thymine dimer lesions generated in UV-irradiated DNA at concentrations as low as 2.5×10^{-10} M (Section 6.4).

The sensitivity achieved in the immunoassay protocol with chemiluminescent detection used here is comparable with

that used by researchers elsewhere. The detection limits for the chemiluminescent assay of ATP and NADH are reported to be 10^{-10} mole and 10^{-16} mole repectively[178]. Application of chemiluminescence in immunology has allowed rabbit IgG[191] and hepatitis B surface antigen[192] to be monitored in a solid phase immunoassay with chemiluminescent detection. However, these latter two assays had the disadvantage of non-specific binding of serum components (e.g. haemoglobin, cytochrome) which can affect the sensitivity of the assay[181].

To obviate the problems associated with binding of non-specific components, Schroeder, Hines et al.[181] monitored hepatitis B surface antigen using a solid phase immunoassay where the antigen/antibody components were bound to the surface of conventional PVC microtitre (ELISA) plates. The protocol allowed for wash steps which eliminated interference from serum components during assay. A luminometer added peroxide to initiate chemiluminescence and provided an automated output of light generated at 10 second intervals. The technique allowed for 0.1 picomol/litre of antigen to be detected.

If the protocol followed by Schroeder, Hines et al. is compared with that used in this work, then, in essence, the same principles have been applied to the quantitation of thymine dimer. Several wash steps were included in the immunoblotting portion of the protocol to reduce the amount of non-specific binding occurring at unreacted portions of the membrane. However the means of quantitation between the method of Schroeder et al.[181] and the work presented here was different. Visual analysis of the immune complexes was used in this research to identify a darkened spot on the luminescence film against the background interference. As will be shown in Section 6.3, where a calibration graph of cut-off antibody dilutions versus dimer concentrations was constructed, use of a luminometer attached to a chart

recorder allowed, in some cases, more sensitive detection of the cut-off dilutions. It thus appears desirable to use a luminometer to confirm cut-off dilutions which may be at the limit of sensitivity of the chemiluminescent detection system.

Despite precautions being taken to minimize background interference (use of a blocking solution and several wash routines), non-specific binding to the membrane was not an easy factor to control. Apart from non-specific binding, background interference can possibly be attributed to the way in which the membranes were washed, as well as the age of the luminescence film. In the former case, between 6 and 9 membranes were washed together in a plastic box using PBS-Tween as the wash solution. The box was placed on a shaking waterbath and the membranes washed by the PBS-Tween solution flowing over them in a backwards and forwards motion. It is possible that this routine could result in some membranes not coming into contact with wash solution, especially if two or more membranes adhered to one another during washing. Thus removal of serum components (causing the high background interference) would not be effected, resulting in very little discrepancy between a darkened immune complex and background interference on the luminescence film. As a suggestion for workers in this field, an improvement could be made by reducing the number of membranes per box, or enclosing each membrane in a glass Polytop vial containing wash solution as was done for membrane incubation with the primary and secondary antibodies. However, such a step is extremely tedious and time consuming, considering that 12 to 18 membranes were treated in a single protocol and each membrane was washed approximately 12 times during the protocol.

The protocols which yielded the strongest immune complex signal against the clearest background were those which utilized fresh luminescence film stored in a refrigerator.

As was outlined in Section 5.2.2.3.2, the luminescence film decayed over a 4 to 6 month period especially if it was maintained at room temperature. Initially the entire film became darkened prior to developing and printing and thereafter no discernable immune complexes were detected on the film. It was thus imperative to check the background signal of the luminescence film and buy fresh film whenever necessary.

A further consideration in viewing the antigen/antibody complexes on the luminescence film is whether the outer ring of the complex or the inside of the complex be viewed to determine if the complex is present at a specific antibody dilution. At the lower DNA concentrations (< 0.025 mg/ml DNA) the antigen blots did not appear to dry and bind to the membrane in the circular shape that the antigen was delivered to the membrane surface. Instead, lower surface tension (in the less concentrated DNA samples) appeared to cause the antigen blot to spread out, resulting in uneven drying of the blot on the membrane. The problem was compounded if the membrane itself dried erratically so that a non-symmetric blot usually with a darkened rim and of varying intensity was observed on the luminescence film, making detection and quantitation extremely trying. With a fair degree of success, this problem was averted by viewing the most symmetrical blot over a range of 2 or 3 blots produced using identical conditions and incubation times. The result was confirmed using the scanning densitometer (Section 6.4) to confirm or negate a signal against background interference.

Thus the sensitivities afforded by both immunoblotting and ECL together allow the quantitation of thymine dimer in UV-irradiated DNA at concentrations as low as 2.5×10^{-10} M. It therefore appears possible that the immunoassay technique described in this thesis will provide the sensitivity called for in the planned extension (in this laboratory) of

kinetic and mechanistic studies of thymine dimer quantitation to *in vivo* cellular systems. In this context, pyrimidine dimers generated at sub-lethal UV fluences have been detected by Wani, D'Ambrosio and Alvi[162] who demonstrated the quantitation of these dimers in human cells by immuno-slot blotting analysis. Fibroblasts were irradiated with a germicidal lamp (254 nm), the DNA extracted (phenol, chloroform) and the DNA used in an immuno-slot blot assay as proposed by Nehls et al.[84]. (Immuno-slot blot assays utilize a sophisticated piece of equipment to deliver the required volume of antigen to the membrane surface through a slot which ensures a constant shape of antigen on the surface.) Results indicate that irradiation with light of a 0.5 J/m^2 fluence allowed 20 ng of irradiated DNA to be detected and hence adds support to the applicability of this immunoblotting technique for the quantitation of low concentrations of DNA found in cellular systems. In these laboratories, quantitation of dimers in cellular systems was initiated by irradiation of *E.coli* bacteria in the presence of acetophenone[51]. Although problems were experienced in absorption of acetophenone by the bacteria, this irradiation substrate is suitable for the work presented here, the applications of which are described in Section 6.8.

In order to detect low concentrations of thymine dimer, it is imperative to establish a calibration graph of cut-off antibody dilutions versus thymine dimer concentrations. Thus, the following Section (6.3) discusses aspects of a calibration graph that was constructed to allow the quantitation of dimer yields at levels undetectable by the HPLC currently employed in these laboratories.

6.3 CONSTRUCTION OF A CALIBRATION GRAPH FOR THYMINE DIMER QUANTITATION

This research has been directed towards the development of

an immunoassay technique for the quantitation of the most important UV-photoproduct, the cis-syn thymine dimer. As was discussed in Chapter 5, the immunoassay protocol of immunoblotting was developed in conjunction with enhanced chemiluminescence to allow the detection and quantitation of thymine dimer, this technique was then used successfully in determining antibody titres in rabbit serum during and after a series of injections of the UV-irradiated DNA antigen into laboratory animals (Section 6.2). However, if this immunoblotting protocol is to be used in detecting thymine dimer in cellular systems *in vivo*, then the technique must be sufficiently sensitive to detect extremely low levels (μg/ml) of thymine dimer. With this in mind, a calibration graph of antibody cut-off dilutions versus known dimer concentrations was constructed so that the dimer concentration of an unknown antigen sample (i.e. UV-irradiated DNA) could be obtained by determining the observed cut-off dilution and relating it (through the calibration graph) to dimer concentrations.

Currently in these laboratories dimer concentrations in UV-irradiated DNA are measured by HPLC. Thus the HPLC was used to measure dimer concentrations required to construct the calibration graph of cut-off antibody dilutions versus dimer concentrations.

In order to do this, a range of DNA-acetophenone-PBS solutions of varying concentrations were prepared as outlined in Section 2.8.2.5 and irradiated using the HBO 500W/2 short arc mercury lamp (Section 2.8.1.2). The DNA solutions were irradiated to reach maximum or near maximum dimer yields (1.0 mg/ml DNA for 10 hours and 0.5 mg/ml for 6 hours). A second 0.5 mg/ml DNA sample was irradiated immediately after the first one. This second UV-irradiated DNA sample was diluted as follows: after transferral to a Durham tube, a 200 μl aliquot of the 0.5 mg/ml UV-irradiated DNA was transferred to a clean and dry Durham

tube. Addition of 200 μl of PBS to the second Durham tube diluted the DNA sample to 0.25 mg/ml in a 400 μl volume. Using the remaining 200 μl aliquot of the 0.5 mg/ml UV-DNA solution as the "stock", the following dilutions were performed:

Remove 80 μl 0.5 mg/ml UV-DNA + 320 μl PBS . . 0.1 mg/ml
Remove 40 μl 0.5 mg/ml UV-DNA + 360 μl PBS . 0.05 mg/ml
Remove 20 μl 0.5 mg/ml UV-DNA + 380 μl PBS . 0.025 mg/ml
Remove 10 μl 0.5 mg/ml UV-DNA + 390 μl PBS 0.0125 mg/ml

Although lower DNA concentrations were prepared (and will be discussed later in this section), the abovementioned concentrations of 1.0 mg/ml to 0.0125 mg/ml were used as a starting point in constructing the calibration graph. It is important to note that all volumes correspond to the original 400 μl of sample that was irradiated. Each 400 μl sample of a different DNA concentration was then split: 200 μl for analysis by HPLC and 200 μl for analysis by immunoblotting. Such a step was necessary to ensure that the light intensity (and hence energy) impinging on the DNA was identical for each aliquot to be analysed by the two different techniques.

6.3.1 HPLC ANALYSIS

The calibration graph of dimer concentration versus integrated peak area constructed in Section 3.2.3 was specific for the chromatographic parameters (column, eluant, flow rate, injection volume) employed. Hence, for this calibration graph to be useful, the same parameters were used in determining dimer yields of the 1.0 mg/ml to 0.0125 mg/ml DNA 200 μl samples. Despite the fact that only 200 μl of each sample was available for HPLC analysis (as opposed to 400 μl elsewhere in this work), this did not prove to be a problem during acid hydrolysis of the samples. The volumes of reagents outlined in Section 3.1.1

were halved, whereas the acid hydrolysis reaction and centrifugation were still used at 1 h and 15 min respectively. In this manner, 30 μl of each hydrolysate was injected into the HPLC onto an Ultracarb 5 ODS 30 C_{18} reverse phase column and Milli-Q water (pH 6.0) mobile phase at a 0.7 ml/min flow rate. Multiple injections of a particular DNA sample were performed on the HPLC. Dimer peak areas were integrated at 220 nm and the calibration graph (Section 3.2.3) used to calculate dimer yields. Table 6.4 illustrates the DNA concentrations analysed as well as the resulting dimer concentrations. These results are represented graphically in Figure 6.5. This graph confirms the linearity between DNA concentration and thymine dimer concentration in UV-irradiated DNA observed by others in these laboratories[50,54].

[DNA] / mg/ml	[Dimer]* / M	Range of [Dimer]†/M
1.0	3.722×10^{-5}	$(3.443$ to $4.088) \times 10^{-5}$
0.5	1.926×10^{-5}	$(1.894$ to $1.956) \times 10^{-5}$
0.25	8.840×10^{-6}	$(8.500$ to $9.940) \times 10^{-6}$
0.1	4.621×10^{-6}	$(4.090$ to $5.030) \times 10^{-6}$
0.05	2.013×10^{-6}	$(1.660$ to $2.360) \times 10^{-6}$
0.025	-	-

*. Average dimer yield

†. Range of dimer yields obtained by multiple analysis of the UV-DNA sample.

Table 6.4. Cis-syn thymine dimer yield as a function of DNA concentration, [Acp] = 2×10^{-2} M, 1 mg/ml DNA = 10 hours iradiation and 0.5 mg/ml to 0.025 mg/ml = 6 hours irradiation.

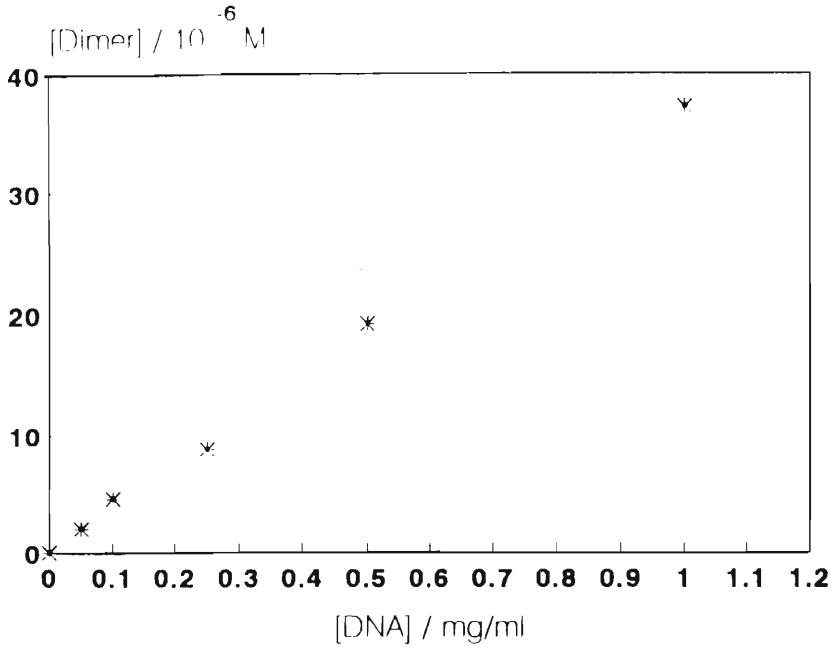
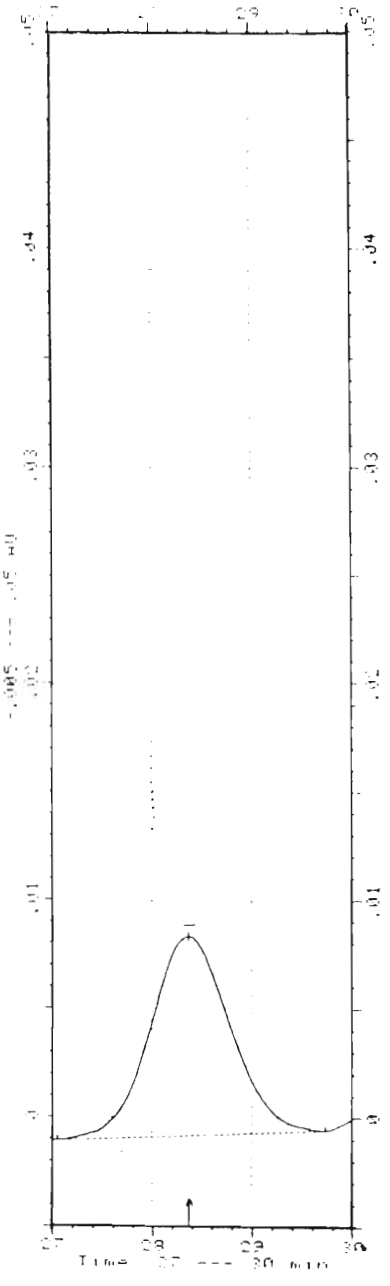


Figure 6.5. Cis-syn thymine dimer yields as a function of DNA concentration, [Acp] = 2 x 10^{-2} M, 1.0 mg/ml DNA = 10 hours irradiation and 0.5 mg/ml to 0.025 mg/ml DNA = 6 hours irradiation.

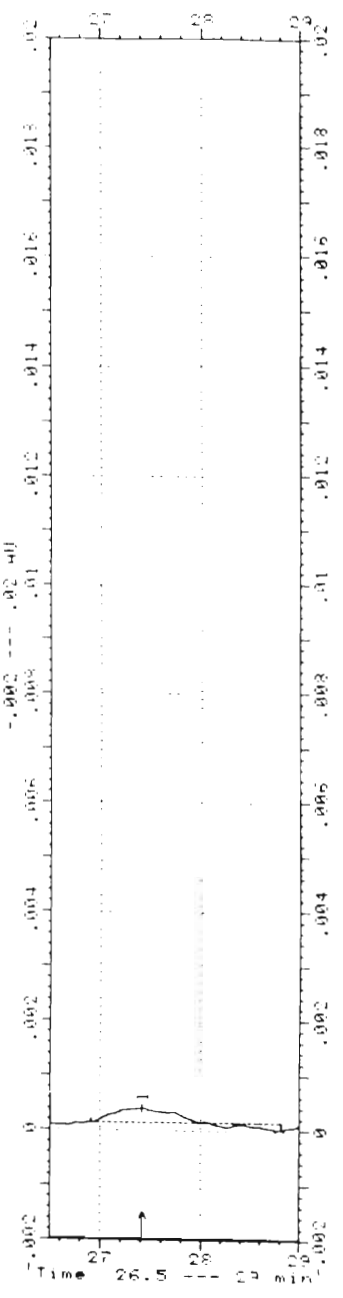
For the 0.5 mg/ml and 1.0 mg/ml DNA samples, a 0.05 AUFS (Absorbance Units Full Scale) value on the y-axis of the chromatogram was used. For the other DNA concentrations (0.025 mg/ml and lower) 0.02 and 0.01 AUFS were employed for the y-axis. These two last mentioned scales show significant signal-to-noise ratios during analysis. Thus while it was difficult to analyse low concentrations of dimer (because the sensitivity threshold of the instrument was being reached), the situation was worsened because of a drifting baseline with significant signal-to-noise ratio.

This made analysis extremely difficult. Figure 6.6. illustrates the peak integration area for 1 mg/ml DNA, as opposed to the peak used by the computer to integrate the dimer area for the 0.05 mg/ml DNA. In the latter case, the peak is not significantly different from the baseline noise. To minimize this instability as far as possible, the instrument was stabilized for approximately 1 hour between injections of DNA hydrolysate.

When the 0.05 mg/ml DNA sample was analysed by HPLC, the dimer peak did not give its characteristic UV spectrum. However, after the "Spectrum Analysis" file of the HPLC programme (loaded onto the attached NEC APC11 computer) was used, the dimer was identified according to its retention time and UV spectrum. The "Integration" file then allowed a peak area to be calculated at the particular retention time. These observations can be seen in Figure 6.7. for the analysis of 0.05 mg/ml DNA. Using the same technique, the 0.025 mg/ml DNA sample was analysed by HPLC, but a UV spectrum was not identified using the "Spectrum Analysis" file. So the thymine dimer generated in this sample of DNA was not detectable by HPLC. Thus cis-syn thymine dimer of approximately 2×10^{-6} M, generated after 6 hours UV irradiation of a 0.05 mg/ml DNA sample was detected by the Waters 990 photodiode array detector.



1.0 mg/ml DNA



0.05 mg/ml DNA

Figure 6.6. Cis-syn thymine dimer peak integration for 1.0 mg/ml DNA versus 0.05 mg/ml DNA.

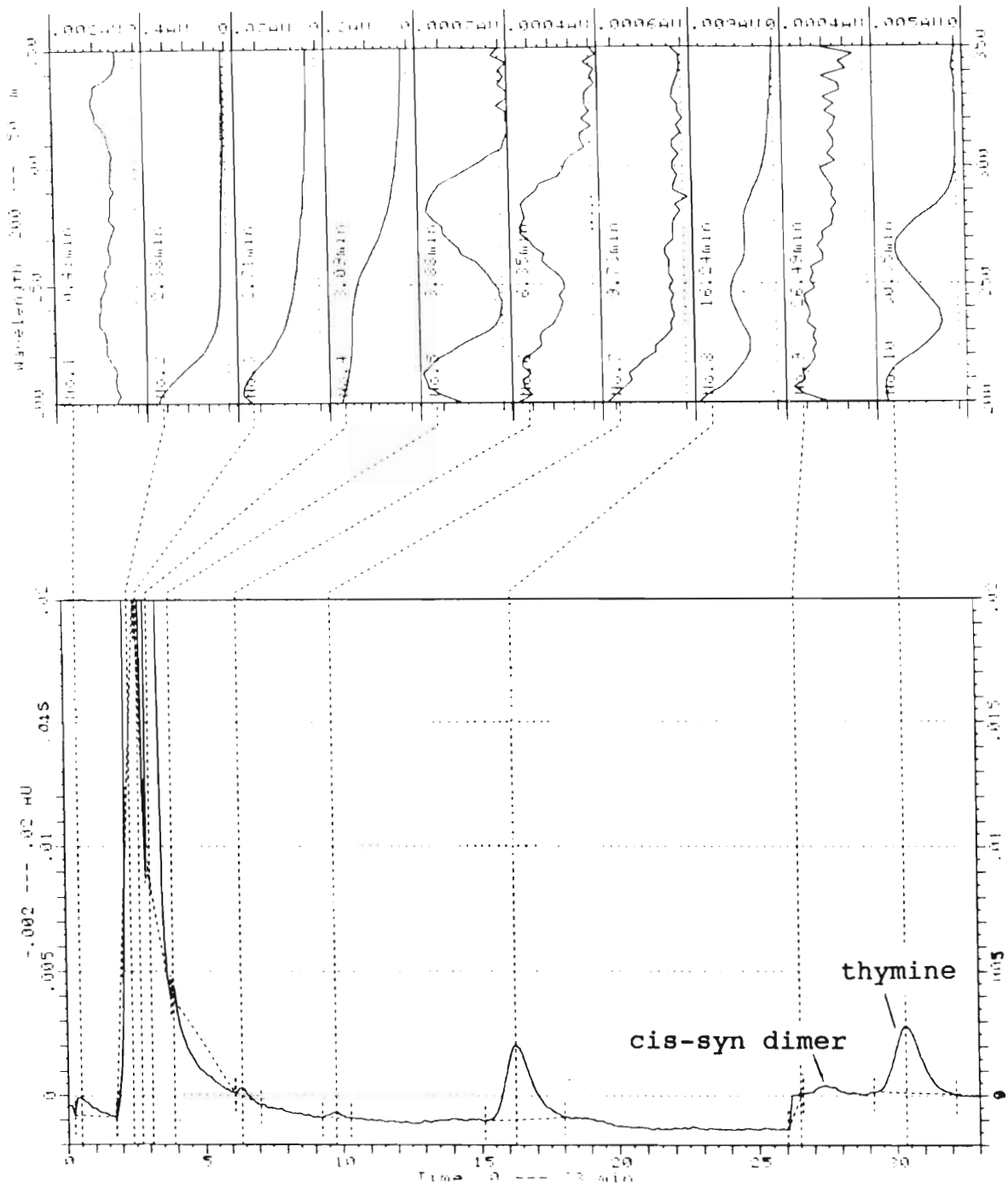


Figure 6.7. HPLC chromatogram showing separation of a 0.05 mg/ml DNA hydrolysate illustrating specifically the cis-syn dimer peak undetectable by the Waters 990 photodiode array detector.

It was not important to determine the absolute detection limit of the instrument, as the main crux of the thesis involves the determination of dimer yields (by immunoblotting) at DNA concentrations below the HPLC detection limit. (As mentioned elsewhere previous workers in this laboratory[54] could only examine acetophenone photosensitized irradiations of DNA at concentrations greater than 0.1 mg/ml DNA.)

The next section discusses the immunoblotting protocol (antigen and antibody dilution preparation) used in this work to determine dimer concentrations in UV-irradiated DNA at levels undetectable by the HPLC. The use of scanning densitometry will be outlined as a confirmatory technique to detect antigen/antibody complexes on the luminescence film after ECL.

6.3.2 IMMUNOBLOTTING WITH ENHANCED CHEMILUMINESCENCE QUANTITATION

For HPLC purposes the UV-irradiated DNA was acid hydrolysed to break down the DNA to its component parts prior to analysis. For immunoblotting purposes, this same UV-irradiated DNA was the antigen in the protocol developed in Chapter 5. As was outlined in that chapter, the DNA was denatured (in addition to being irradiated) before being complexed to methylated bovine serum albumin and Freunds Incomplete Adjuvant, and then injected into rabbits. However, when the UV-DNA was used as the antigen in the immunoblotting protocol, it was merely denatured before being bound to the Immobilon-N membrane. Such a step helped prevent antibodies generated against MBSA and Freunds Incomplete Adjuvant responding to the immmobilized antigen.

Antigen preparation in the immunoblotting protocol involved denaturation of the 200 μl aliquots of varying DNA concentrations by heating for 10 minutes at 100°C, followed

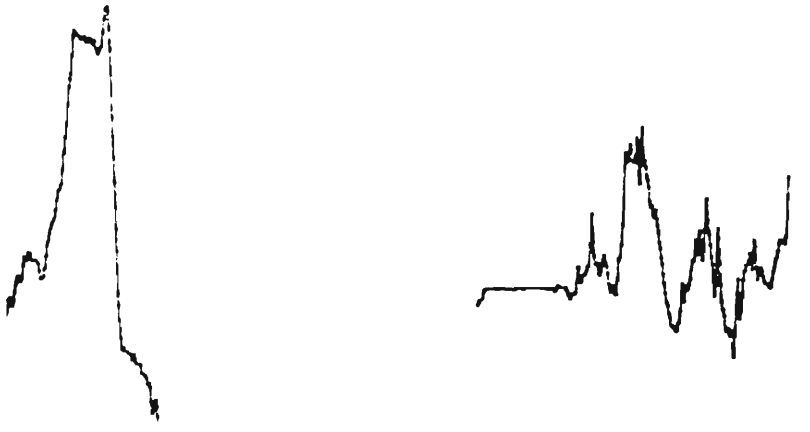
by cooling on ice for 10 minutes. All solutions required for immunoblotting analysis were prepared fresh. Because the construction of the calibration curve necessitated the use of much serum sample, the i/v T (intravenous terminal) serum was used (approximately 50 ml of serum was obtained after cardiac puncture, in comparison with 0.5 ml to 1.0 ml serum obtained during a routine bleed of the rabbit) and diluted for this purpose. For detection new X-ray luminescence film as well as developer and fixer were acquired and stored in a refrigerator (4°C) to prolong their lifespan.

The ECL detection solutions supplied by Amersham were tested to ensure that blue light of 425 to 430 nm was generated. This was performed by adding 500 µl of each detection solution to the same 5 ml glass beaker. With the lights switched off, 3 µl of stock secondary antibody conjugate was added to the detection solutions and the contents of the beaker swirled by hand. The presence of the blue light confirmed the detection solutions to be generating and catalysing the light emission signal.

The i/v T serum which was frozen was brought to room temperature. Fresh serial dilutions of this serum were prepared as outlined in Section 5.2.1.7.5 in clean and dry Polytop 12 ml glass vials. Using the 200 µl sample of the 1 mg/ml irradiated and denatured UV-DNA as the antigen, the immunoblotting protocol was performed as summarized in Section 5.2.1.8. Primary antibody dilutions of 1:400 to 1:12150 were used as a starting point, and only two 5 µl aliquots of antigen were spotted onto the Immobilon-N membrane to conserve sample. (If the sample had run out, more would have had to be irradiated and once again analysed by HPLC.) The ECL detection protocol (summarized in Section 5.2.2.4) was performed on the antigen/antibody complexes immobilized on the membranes. Results indicated that the cut-off dilution of the antibody sample for the

1.0 mg/ml DNA antigen was not reached with a 1:12150 dilution of the serum.

Hence, further dilutions of the i/v T serum (1:20000, 1:30000, 1:45000 and 1:67000) were prepared and the immunoblotting protocol was performed using these dilutions and the 1 mg/ml UV-DNA antigen. ECL detection and analysis indicated that the antigen/antibody complex was still present on the X-ray film. Further dilutions of 1:80000, 1:90000, 1:100000 and 1:110000 were used in the same immunoblotting protocol. Visual analysis of the X-ray film indicated the 1:80000 and 1:90000 and possibly the 1:100000 dilution were present as an antigen/antibody complex on the film. Because these results were integral to the thesis, the protocol was repeated with the 1:80000 to 1:110000 dilutions of serum to obtain multiple X-ray films of the potential 1:100000 cut-off dilution of antibody using a 1 mg/ml antigen. In addition, the same protocol was performed using PBS as the antigen to check that it was the solute (UV-irradiated DNA) and not the solvent (PBS) causing a darkened spot on the X-ray film. No darkening of the film was observed visually using the PBS as antigen. Those X-ray films containing the "possibly visible" antigen/antibody complexes were examined using scanning densitometry. A GS 300 Transmittance/Reflectance scanning densitometer (Hoeffer Scientific Instruments, San Fransisco) attached to a Varian 4270 Chart Recorder/Integrator (both on loan from the Department of Biology at the University of Natal, Durban) was used to scan the X-ray film over the darkened areas. Although problems were experienced in scanning against a relatively dark background of the X-ray film, Figure 6.8 illustrates the 1:80000 and 1:1000000 dilutions to be present against the background noise, the height of the signal being proportional to the intensity of the spot and hence proportional to the concentration of antigen bound to the membrane. The 1:110000 showed no difference in comparison with the background and the 1:100000 was



1:80000 dilution 1:100000 dilution

Figure 6.8. Chart recorder signals of 1:80000 and 1:100000 dilutions of crude serum used in an immunoblotting protocol to determine the cut-off dilution of the 1.0 mg/ml DNA antigen.

tentatively assigned to the cut-off dilution for the 1 mg/ml DNA sample. It is important to add though, that any value between 1:100000 and 1:110000 could possibly be the correct one, but time did not permit the preparation of a series of dilutions between these values, nor the performance of the lengthy protocols required.

In the same manner that has been described above, individual 0.5 mg/ml, 0.1 mg/ml, 0.05 mg/ml 0.025 mg/ml and 0.0125 mg/ml DNA samples were irradiated and used as the antigen in the immunoblotting protocol with ECL detection of the immune complexes. Table 6.5. shows the DNA concentrations and cut-off dilutions that were detected by visual inspection of the X-ray film. It also illustrates the cut-off dilutions that were determined using scanning densitometry. A few of these dilutions have been extended using this alternative detection technique. The data is represented graphically in Figure 6.9.

[DNA] / mg/ml	[Dimer] / M	Cut-off dilution (visual)	Cut-off dilution (densi-tometry)
0.50	1.926×10^{-5}	1:45000	1:45000
0.10	4.621×10^{-6}	1:19000	1:19000
0.05	2.013×10^{-6}	1:10000	1:10000
0.025	1.006×10^{-6}	1:5400	1:8100
0.0125	5.030×10^{-7}	1:3600	1:5400

Table 6.5. DNA and dimer concentrations used in the immunoblotting assay together with the cut-off dilutions determined using visual analysis and scanning densitometry of the X-ray films.

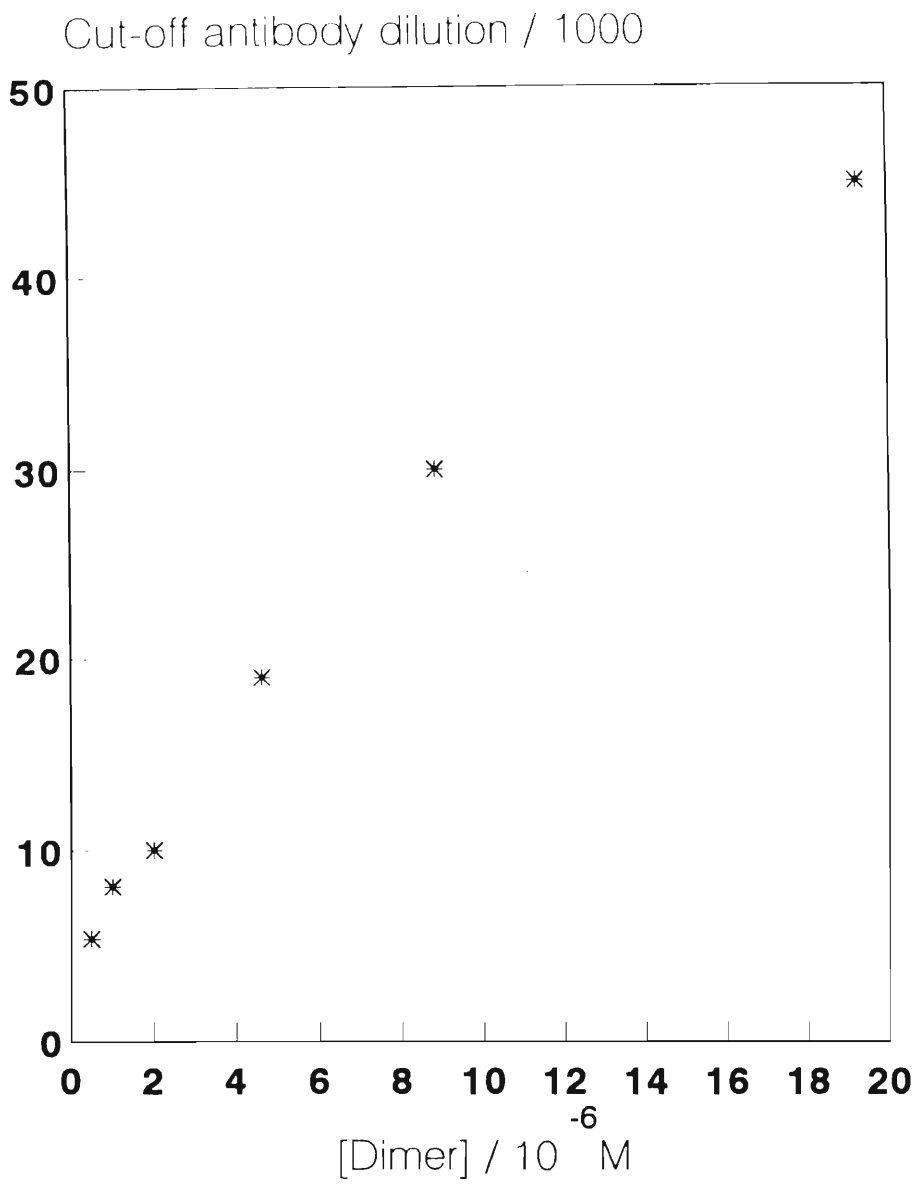


Figure 6.9. Calibration graph of cis-syn thymine dimer concentrations versus cut-off antibody dilutions for DNA concentrations of 0.5 mg/ml to 0.0125 mg/ml.

A further investigation to determine the lowest detection limit of the immunoblotting protocol was carried out. In this case, a single 0.5 mg/ml DNA sample was irradiated for 6 hours and diluted. The assays yielded the cut-off dilutions as shown in Table 6.6. at these lower DNA concentrations.

[DNA] / mg/ml	[Dimer] / M*	Cut-off Dilution
6.250×10^{-3}	2.516×10^{-7}	1:10000
3.125×10^{-3}	1.258×10^{-7}	1:5400
7.812×10^{-4}	3.145×10^{-8}	1:3000
1.953×10^{-4}	7.863×10^{-9}	1:400
3.125×10^{-5}	1.258×10^{-9}	1:50
6.250×10^{-7}	2.516×10^{-10}	1:1

* obtained by dilution of a 0.5 mg/ml UV-irradiated DNA sample.

Table 6.6. DNA and dimer concentrations used in the immunoblotting protocol together with the cut-off antibody dilutions determined using scanning densitometry of the X-ray films.

As was outlined earlier in this section, the X-ray films of the lower DNA concentration as antigen were also examined under a GS 300 Transmittance/Reflectance scanning densitometer to confirm or negate a "possibly visible" antigen/antibody complex on the X-ray film. The dimer concentrations versus cut-off dilutions are represented graphically in Figure 6.10.

If the cut-off dilutions determined by immunoblotting for the entire DNA concentration range (1.0 mg/ml to 6.25×10^{-7} mg/ml) are examined, a distinct anomaly becomes evident. A DNA concentration of 5×10^{-2} mg/ml has a cut-off dilution of 1:10000, while this same cut-off dilution is obtained

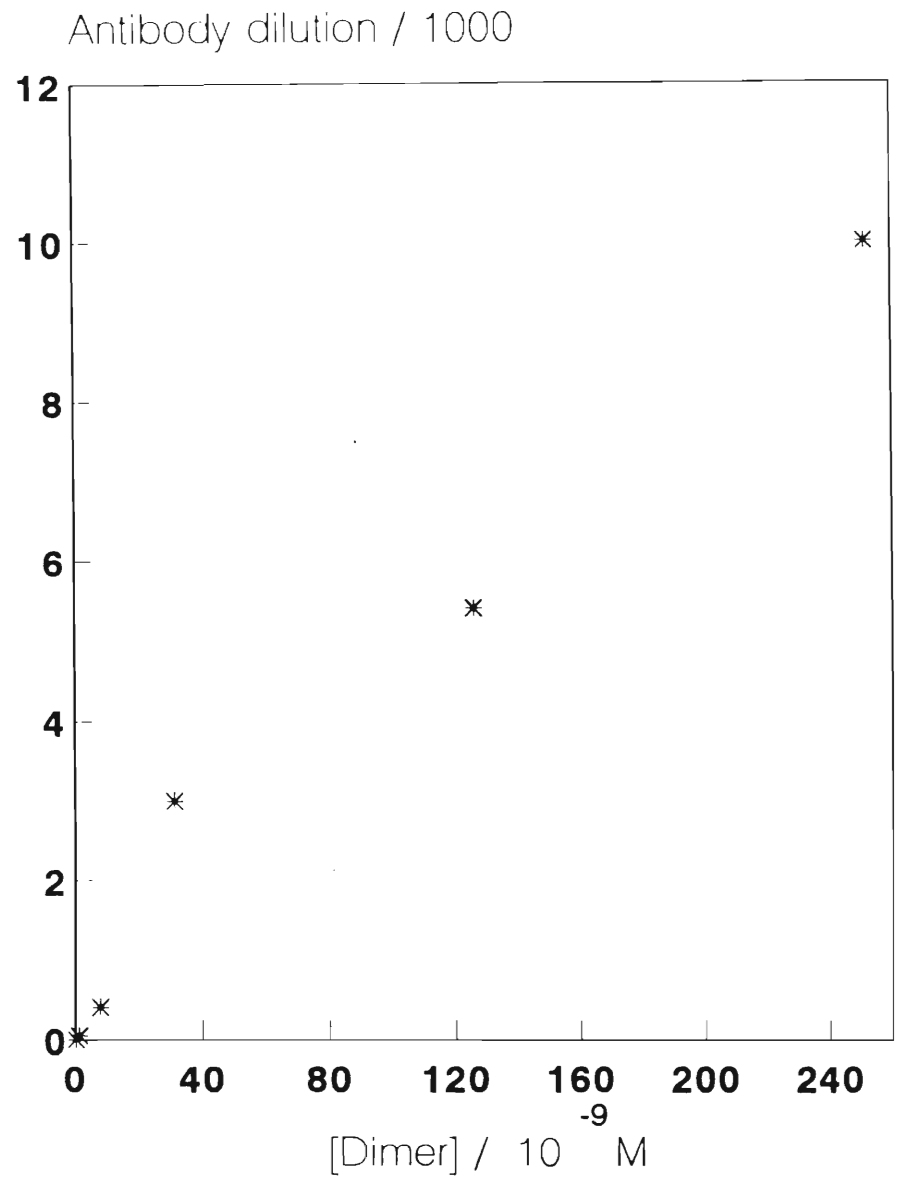


Figure 6.10. Calibration graph of cis-syn thymine dimer concentrations versus cut-off antibody dilutions for DNA concentrations of 6.25×10^{-3} mg/ml to 6.25×10^{-7} mg/ml.

for the 6.25 x 10^{-3} mg/ml DNA antigen. In the same vein, the 1.25 x 10^{-2} mg/ml DNA concentration has a cut-off of 1:5400, while it is also the cut-off for the 3.125 x 10^{-3} mg/ml DNA concentration. Figure 6.11. illustrates the dimer concentration plotted against the cut-off dilutions for the entire range of DNA concentrations used. It was thought that this calibration graph would yield a straight line of dimer concentration versus cut-off dilution since the lower the dimer concentration, the fewer the antibody molecules which would bind to the dimer, hence more dilute serum samples (containing proportionally fewer antibody molecules) would be needed to produce this cut-off dilution observed as a lightening of the antigen/antibody complex on the X-ray film after ECL detection. Clearly, an unexpected feature of the immunoblotting protocol is coming to light.

6.3.3 EXPLANATION OF THE ANOMALY IN THE CALIBRATION GRAPH

As was outlined above (Section 6.3.2), a non-linear calibration graph was obtained (see Figure 6.11) to relate dimer concentrations (determined by HPLC) and cut-off antibody dilutions of antigen/antibody complexes (determined by immunoblotting with ECL detection). The non-linearity of the calibration graph can possibly be explained in terms of a "masking effect" on the Immobilon-N membrane itself. At the higher DNA concentration (e.g. 0.025 mg/ml) the DNA which is acting as the antigen in this protocol, is saturating the membrane. Hence, a layering of antigen is occurring on the membrane surface limiting access of antibody and secondary antibody molecules to every thymine dimer molecule during the immunoblotting protocol. Because a series of reagents are added succesively to the membrane, a bulky antigen/antibody complex (formed after incubating the antigen containing membrane with diluted serum) could restrict access to other antigen/antibody complexes during addition of the secondary

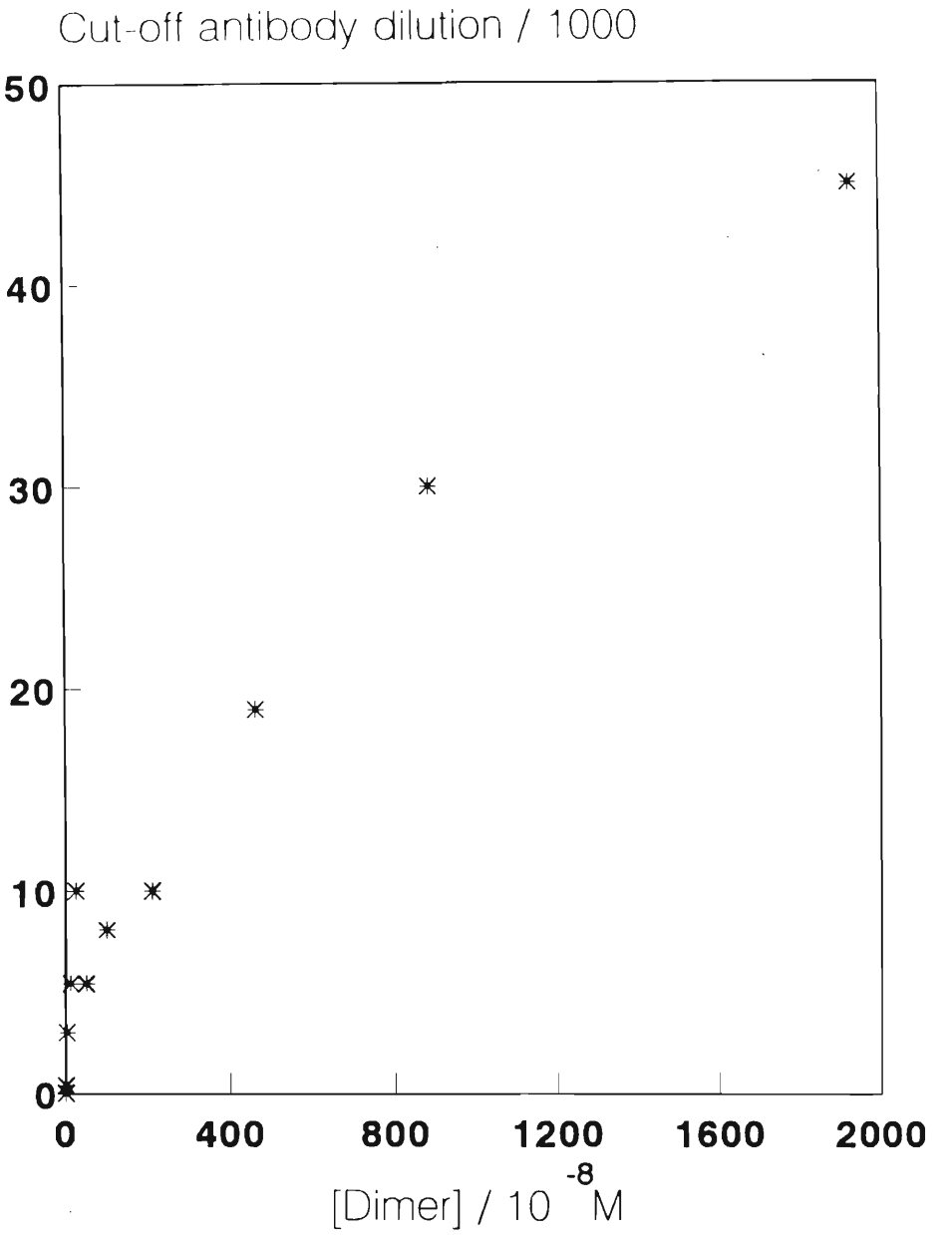


Figure 6.11. Calibration graph of cis-syn thymine dimer concentrations versus cut-off antibody dilutions for DNA concentrations of 5×10^{-1} mg/ml to 6.25×10^{-7} mg/ml.

antibody conjugate (which is itself bulky). Thus steric hindrance and the phenomenon of "site restriction" can explain the non-linearity of the calibration graph.

In terms of the explanation given above, at the lower antigen concentrations (6.25×10^{-3} mg/ml to 6.25×10^{-7} mg/ml DNA) that portion of the membrane that was blotted with antigen is not saturated with the antigen. Hence no, or little, layering of the UV-irradiated DNA on the membrane is occurring. Thus each antigen molecule comes into contact with primary and secondary antibodies, resulting in darker antigen/antibody complexes on the luminescence film. This extends the range of cut-off dilutions (which were observed in Section 6.3.2) and hence affords "increased sensitivity" at these lower DNA concentrations.

In molecular biology applications, ethidium bromide is used to confirm the presence of DNA (or its constituents) in solution or immobilized on membranes (as in this work) by intercalating with the DNA. However, its applications are qualitative in nature and the compound must be used in the dark. Thus, although it could theoretically be used to stain the DNA immobilized on the membrane, it would not reveal whether a certain concentration of DNA was saturating the membrane. However, a means of obviating site restriction of the antigen would be to decrease the volume of UV-irradiated DNA blotted onto the membrane surface. Section 5.2.1.7.2 explained the choice of a 5 μl antigen volume. This could be reduced to 1 or 2 μl, but this modification was not attempted in this work because of time constraints. This does, however, provide a suggestion for future workers.

The following section (6.3.4) discusses the choice of calibration graph needed to convert cut-off antibody dilutions to thymine dimer yields.

6.3.4 ESTABLISHMENT OF THE CUT-OFF ANTIBODY DILUTION VERSUS THYMINE DIMER CALIBRATION GRAPH

To help clarify the discussion which is to follow, Table 6.7. illustrates the dimer concentrations used and the resulting cut-off dilutions determined by immunoblotting for the entire range of DNA concentrations employed.

$[Dimer]_{exp}$ / M	Cut-off Dilution
1.926×10^{-5}	1:45000
8.840×10^{-6}	1:30000
4.621×10^{-6}	1:19000
2.103×10^{-6}	1:10000
1.006×10^{-6}	1:8100
5.030×10^{-7}	1:5400
2.516×10^{-7}	1:10000
1.252×10^{-7}	1:5400
3.145×10^{-8}	1:3000
7.863×10^{-9}	1:400
1.258×10^{-9}	1:50
2.516×10^{-10}	1:1

Table 6.7. Experimentally determined cis-syn thymine dimer yields and cut-off dilutions for DNA concentrations of 5.0×10^{-1} mg/ml to 6.25×10^{-7} mg/ml.

Regression analysis (using the computer programme Statgraphics) was performed on selected and all calibration points. Both simple and multiple linear regression options were used. Simple regression fits the best straight line (given by $y = mx + c$) through the points. Unlike simple regression, multiple regression fits the best straight line through the origin (i.e. $y = mx$). Both procedures use least

squares to estimate the regression model.

Four regression analyses were performed:
1. Regression analysis of the lower region of the calibration graph using the simple linear mode of analysis.
2. Regression analysis of the lower region of the calibration graph using multiple regression.
3. Regression analysis of the entire calibration graph using a simple linear mode of analysis.
4. Regression analysis of the entire calibration graph using multiple regression.

The slope, intercept and correlation coefficients for these regression analyses are given in Table 6.8.

	Slope / dilution M^{-1}	Intercept / Dilution	Correlation Coefficient
1	3.878×10^{10}	438.7	0.9850
2	4.107×10^{10}	0	-
3	2.503×10^{9}	3974	0.9927
4	2.659×10^{9}	0	-

Table 6.8. Slope, intercept and correlation co-efficient data for the four regression analyses performed on the calibration data points.

As will be seen in Section 6.5, substantial antibody production has occurred against non-thymine dimer antigens and this fact must be accounted for by the choice of calibration graph. To this end, the graph which most adequately fits the observation is given in Figure 6.12., where a y-intercept of 3974 has been calculated by the Statgraphics regression analysis programme (Table 6.8.). In this context, it must be noted that analysis 2 and analysis 4 do not allow for non-thymine dimer antibody production

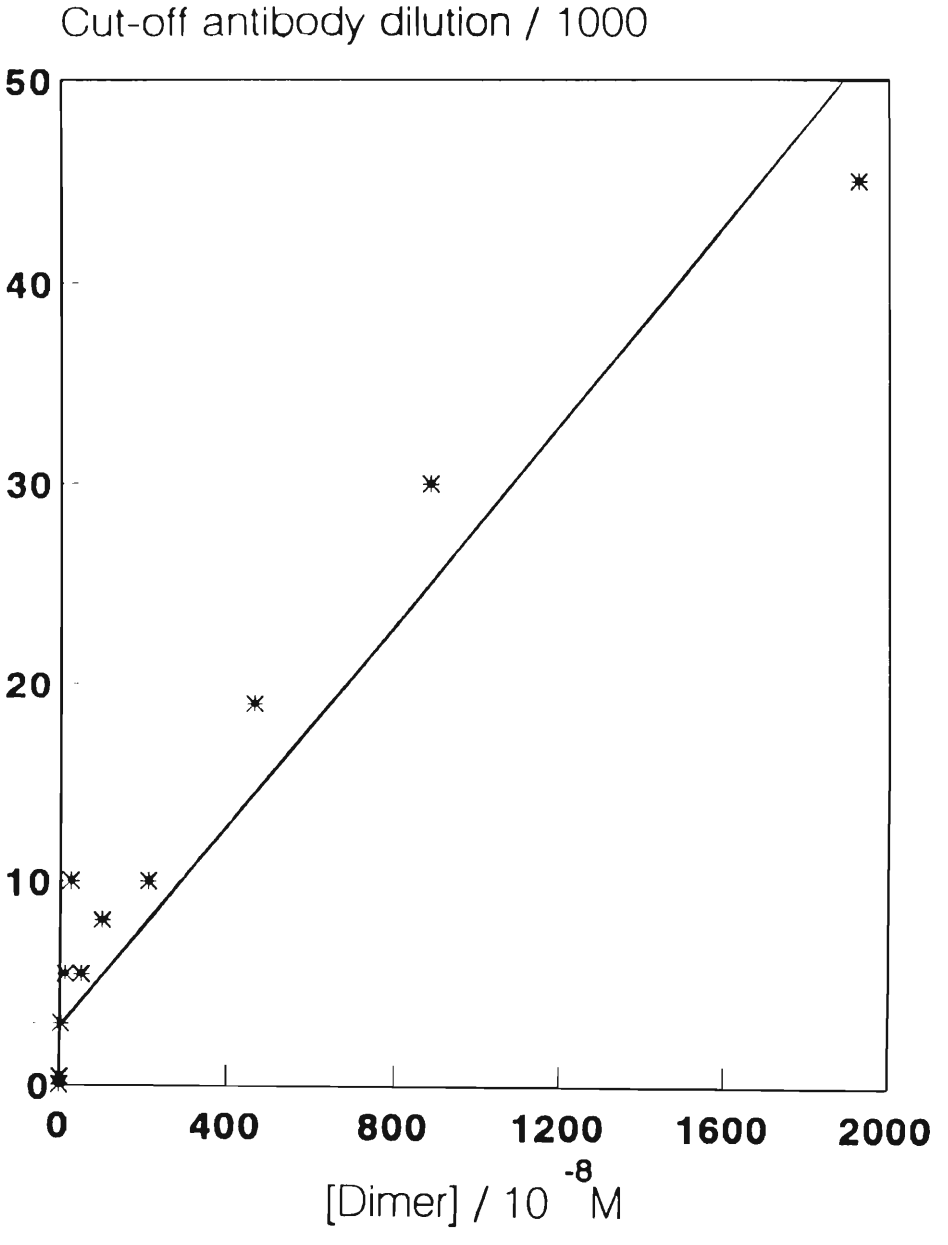


Figure 6.12. Calibration graph used to relate cut-off antibody dilutions to thymine dimer yields.

and therefore do not adequately reflect these observations. Analysis 1 allows non-dimer antibody production of only one tenth that observed in analysis 3 and thus is also excluded.

Thus despite the apparent non-linearity of the calibration graph, one can fit a straight line to this data with a slope of 2.503×10^9 dilution M^{-1} and intercept of 3974. The correlation coefficient of these points was calculated by the programme to be 0.9927. The intercept of 3974 possibly takes account of the antibody production against MBSA and Freunds Adjuvant. It seems logical that it accounts for antibody directed against these components and not the associated DNA components (phosphate-deoxyribose backbone, purine and pyrimidine bases and histone protein) because a constant volume and concentration of MBSA and Freunds Adjuvant were injected into the rabbits during each of the immunization schedules. In contrast, DNA of varying concentrations (1.0 and 0.5 mg/ml) were injected into the rabbits during successive immunization schedules.

Since some antibody production is directed against non-dimer antigen, the relationship between dimer concentration and antibody dilutions can be expressed as

$$D = \frac{k}{A-a}$$

where D is the thymine dimer, k is the slope of the calibration graph relating dimer concentrations to cut-off antibody dilutions (A) and a is that portion of the antibody production directed against non-dimer antigen. The above relationship is based upon the linearity between cut-off antibody dilutions and thymine dimer yields as was established earlier in this section.

Thus

$$\log k(A-a) + \log D = \log k$$

and

$$\log(kA-ka) + \log D = c$$

where c represents a constant. Replacing kA with y and ka with y_o, then

$$\log(y-y_o) + \log D = c$$

Differentiating the above equation leads to

$$\frac{dy}{y-y_o} + \frac{dD}{D} = 0$$

then,

$$\frac{dD}{D} = -\frac{dy}{y-y_o}$$

and

$$\frac{dD}{D} = -\frac{\frac{dy}{y}}{1-\frac{y_o}{y}}$$

where $\frac{dD}{D}$ represents the error in determining dimer yields

and $\frac{dy}{y}$ represents the error in estimation.

More specifically the latter estimates the ability of a naked eye to detect the presence or absence of an immune complex on the luminescence film. If this value can be estimated, then the error in determining the dimer yields can be calculated.

Visual analysis is used to calculate pH measurements when comparing a given reaction colour against a colour pH scale. Assuming it is possible to visually observe a 0.1 pH difference, then the range of values which can be observed is 0.2. Seeing that visual analysis is also used in this work, this value (0.2) was inserted into the final equation.

Using the cut-off dilutions outlined in Table 6.7., the following table provides the relevant errors in calculating the thymine dimer yields based on the cut-off antibody dilutions.

Cut-off dilution	$\frac{dD}{D}$	$\frac{dD}{M}$
1:45000	-0.2194	4.225×10^{-6}
1:30000	-0.2305	2.037×10^{-6}
1:19000	-0.2529	1.168×10^{-6}
1:8100	-0.3319	6.681×10^{-7}
1:5400	-0.3926	3.949×10^{-7}
1:10000	-0.3319	8.351×10^{-8}
1:5400	-0.7573	9.527×10^{-8}

Table 6.9. Table illustrating the error of estimation of dimer yields based on the error in determining cut-off antibody dilutions.

Although dimer concentrations below 1.258 x 10^{-7} M were analysed in this work (correlating to an error of 9.527 x 10^{-8} M), the cut-off dilution of 3974 attributed to non-dimer antibody production does not allow an error to be determined for the 1:3000, 1:400, 1:50 and 1:1 antibody dilutions.

The following section (6.4) discusses the sensitivity of the immunoblotting protocol as used for the quantitation of thymine dimers in UV-irradiated DNA. Thereafter the discussion will extend to the investigation of the specificity of the antibody produced by the rabbits in response to the UV-irradiated DNA antigen.

6.4 SENSITIVITY OF IMMUNOBLOTTING ANALYSIS FOR THYMINE DIMER QUANTITATION

In immunoassays, the question of sensitivity of any particular assay can be monitored in two ways. The first assesses the lowest UV fluence required to bring about detectable dimer quantitation. The other approach examines the mass or concentration of thymine dimer detectable by the assay. Researchers in the field have commonly adopted the former approach in determining the sensitivity limits of their assays. Amongst them are Seaman et al.[74] who used a radioimmunoassay to measure induction and removal rates of thymine dimer in DNA extracted from bacterial and mammalian cells. The sensitivity limit of detection allowed dimers induced by UV fluences of 5 J/m^2 (254 nm irradiation) to be detected. Later workers increased the sensitivity of radioimmunoassay by using plasmid DNA labelled *in vitro* by nick translation with [^{32}P]-deoxyribonucleotide triphosphates as a competitive tracer. Using this method Mitchell and Clarkson[193] detected photoproducts in cellular DNA at UV doses of 2.5 J/m^2.

More recently, the ELISA assay (Leipold et al.[82] and Wani

et al.[124]) was used to measure pyrimidine dimers in DNA, extending the limit of sensitivity of dimers to 1 to 2 J/m^2. However, Wani et al.[162] (1987) detected dimers induced by even lower sub-lethal (0.5 J/m^2) UV doses using immunoslot blot analysis. Recently an ELISA was used to characterize a monoclonal antibody (TDM-2) which recognised dimers induced by UV fluences of 0.5 J/m^2[194].

In this work, however, a UV source of fixed intensity was employed for the irradiation of *in vitro* calf thymus DNA. It is possible that Pyrex filters could have been employed to reduce the UV intensity, but the choice of filter would have been crucial so as not to interfere with the photosensitized irradiations being performed. It was thus easier to approach the question of sensitivity by considering the lowest dimer concentration detectable by immunoblotting with ECL quantitation.

Indirectly, the sensitivity of the protocol employed in this work has already been calculated. Referring to Table 6.6. (Section 6.3.2), the lowest possible dimer concentration detected using immunoblotting is 2.5×10^{-10} M observed after 6 hours irradiation of a sample of DNA of concentration 6.25×10^{-7} mg/ml. Previous members of this research group[54] (working in the 0.1 mg/ml DNA concentration range), detected about 2×10^{-6} M thymine dimer with 2×10^{-2} M acetophenone (as in this work) and a 1 mm Pyrex filter (unlike the 10 mm Pyrex filter used here) after approximately a half hour irradiation.

As mentioned in Section 6.3, the sensitivity threshold of the Waters 990 photodiode array detector for dimer detection lay between 0.05 mg/ml DNA (where the dimer was detected at 2.10×10^{-6} M) to 0.025 mg/ml DNA (where the dimer was not detected) after 6 hours irradiation of the DNA sample. If an absolute sensitivity threshold of dimer detection at 0.05 mg/ml DNA after 6 hours irradiation is

assumed, together with the immunoblotting limit of 6.25 x 10^{-7} mg/ml DNA, then immunoblotting is approximately 8000 times more sensitive than the HPLC used in these laboratories. If an absolute sensitivity threshold of dimer detection in 0.025 mg/ml DNA is assumed after 6 hours irradiation, then immunoblotting is about 4000 times more sensitive than the detection threshold of the UV photodiode array detector used in this work.

The following section (6.5) considers the specificity of the antibody generated by the rabbit immune system.

6.5 SPECIFICITY OF THE ANTIBODY GENERATED BY THE RABBIT IMMUNE SYSTEM

Researchers who have generated anti-DNA antibodies in laboratory animals have usually undertaken a detailed examination of the antigenic specificity of the antibody produced. Results indicate that the antigenic determinant of antibodies generated against UV-irradiated DNA is the cyclobutane pyrimidine dimer as suggested by shortwave photoreversal[71], enzymatic photoreactivation of the UV photoproducts[72,73,196] and correlation of the antigenic activity with expected or chromatographically measured repair kinetics of dimers[74,195].

The majority of the sera tested in the abovementioned examples showed specificity for the thymine dimer, a few showed specificity for the cytosine-cytosine dimer[71,124,193,196] while the majority were not tested for their specificity to cytosine-thymine dimers or 6-4 photoproducts. At the beginning of the 1980's only the serum produced in the experiments of Strickland and Boyle[198] showed specificity for thymine cyclobutyl dimers and showed no response when tested against cytosine-thymine, cytosine-cytosine, 6-4 photoproducts and other non-dimer photoproducts. However, these researchers were

experimenting with monoclonal antibodies (Section 1.2) producing IgG molecules which recognized adjacent thymine residues in single stranded DNA or synthetic oligonucleotides with a high degree of discrimination.

In 1991 Mizuno et al.[168] developed a monoclonal antibody (TDM-1) which bound to 313 nm UV-irradiated DNA in the presence of acetophenone. A monoclonal antibody recognizing 6-4 photoproducts was developed by Matsunaga et al.[197].

Specificity of a particular anti-UV-DNA serum can be enhanced by removing non-specific antiserum by pre-adsorption with single stranded DNA. Seaman et al.[174] report that pre-adsorption as mentioned above, removed non-specific binding to single stranded DNA without reducing specific (UV single stranded DNA) binding. However, two other studies[195,198] report that anti-UV single strand DNA activity could not be separated from cross-reactivity with undamaged single stranded DNA. These results suggest that in the former case, two different antibody populations were present in the antiserum; one specific for UV single stranded DNA and the other specific for single stranded DNA. In the latter case, a single antibody population with high specificity for UV single stranded DNA and low specificity for single stranded DNA was present.

The investigations noted above allude to the heterogenous nature of the antigenic determinant and the antibodies produced in response to them. In this work UV-DNA complexed to MBSA and Freunds Incomplete Adjuvant was injected into rabbits. Apart from responding to the thymine dimer, the rabbit immune system would also have produced antibodies to the MBSA protein, Freunds Adjuvant, a small concentration of acetophenone and histone protein ($< 3\%$) associated with the DNA as well as alien sequences of calf thymus DNA. As was mentioned in Section 1.2, antibody production to thymine dimer occurs primarily in response to

conformational changes induced by dimerization, rather than the dimers themselves.

To investigate the specificity of the antibody containing serum in this work, a series of immunoblotting protocols were performed using various irradiated and/or denatured DNA samples as the antigen in the protocol. In the routine protocols that were performed in this work, irradiated and denatured DNA was used as the antigen. Hence, the double stranded calf thymus DNA was separated (by denaturation) allowing maximum exposure of the thymine dimer to the rabbit immunocompetent cells. As has already been mentioned, a 1 mg/ml DNA (irradiated and denatured) gave a 1:1000000 cut-off dilution in the immunoblotting protocol. The same protocol was performed using using 1 mg/ml DNA as the antigen. However, the DNA that was used was not irradiated but was denatured prior to use in the protocol. This implied that the DNA strands were separated, allowing antibody production against the DNA deoxyribose-phosphate backbone, the purine and pyrimidine bases and any histone protein. Seeing that the same serum was used in each part of the protocol, antibody production of MBSA and Freunds Adjuvant was identical in each case.

Initial results indicated a cut-off dilution of between 1:55000 and 1:60000. Further investigations confirmed 1:56000 and 1:58000 dilutions being present as antigen/antibody complexes for the s/c 6 (subcutaneous injection of antigen, sixth bleed) and i/m 6 sera used in this assay. The cut-off dilution was thus taken to be 1:60000 which was confirmed using the scanning densitometer as before.

Thus approximately 60% of the antibody generated by the rabbits were directed against DNA constituents. By implication approximately 40% of the antibody production was directly produced against the thymine dimers or

conformational changes induced by the dimerization process.

The following sections (6.6 to 6.8) turn to examine the validity of the previously developed kinetic mechanism for the formation of thymine dimers in calf thymus DNA[54].In previous work the lowest DNA concentration used to test the mechanism was 0.1 mg/ml DNA. Here lower DNA concentrations were used to test the mechanism.

6.6 VALIDITY OF THE PREVIOUSLY DETERMINED MECHANISM AT 0.025 mg/ml DNA

This research is aimed at developing an immunoassay technique to detect thymine dimer concentrations at levels below the sensitivity threshold of the Waters 990 photodiode array detector currently used in this laboratory. To this end, an immunoblotting protocol was established and developed together with the technique of enhanced chemiluminescence for the detection and quantitation of thymine dimer lesions. The majority of the development work for this immunoassay was performed at DNA (and hence thymine dimer) concentrations detectable by HPLC. When the technique was established, DNA concentrations yielding dimer concentrations below the sensitivity limits of the HPLC were tested to see whether immunoblotting provides a more sensitive means of detection over HPLC analysis. The results of this investigation are manifest in the calibration graph data, the details of which indicate that dimer concentrations as low as 2.5×10^{-10} M were detected.

Previous members of this research group[50,54] used HPLC routines for quantitation purposes and used the HPLC results together with chemical and actinometric data to propose mechanisms for photosensitized dimerization for the various thymine containing systems (aqueous thymine, thymidine, thymidylyl-3',5'-thymidine, calf thymus DNA and

plasmid DNA) under investigation. Thomas[54] examined the kinetics of the acetophenone photosensitized irradiation of calf thymus DNA at 0.1 mg/ml to 10.0 mg/ml DNA using both 2×10^{-2} M and 4×10^{-2} M acetophenone. The aim of the work presented in the following sections is to examine the validity of this previously developed kinetic mechanism for the formation of thymine dimers in calf thymus DNA at concentrations lower than those which could be examined using HPLC techniques.

DNA of 0.025 mg/ml concentration was irradiated for varying lengths of time. The irradiated DNA was used as the antigen in immunoblotting analyses after which the cut-off dilutions were correlated to thymine dimer concentrations using a calibration graph (Section 6.3.4). These values together with the mechanism proposed by Thomas[54], were used in the programme CAKE to obtain simulated thymine dimer yields. Support for the mechanism was found by comparing experimental (determined by immunoblotting) and simulated (determined by CAKE) dimer yields.

6.6.1 DNA IRRADIATION AND ANALYSIS BY IMMUNOBLOTTING

The DNA concentration which would give dimer yields undetectable by HPLC was chosen for testing the kinetic mechanism of photosensitized dimerization of thymine in DNA. For this a DNA concentration of 0.025 mg/ml was chosen. At this concentration the antigen/antibody complexes produced in the protocol would be dark and thus hence easier to analyse.

A 0,025 mg/ml DNA solution was prepared as outlined in Section 2.8.2.5 and 400 μl aliquots of the solution were irradiated for varying lengths of time (0.5, 1.5, 2.5, 3.8 and 6 hours). The maximum irradiation time of 6 hours was chosen to correlate with the 6 hour irradiation that the 0.5 mg/ml DNA sample (Section 6.3.1) was subjected to. In

addition, the Blak-Ray J-221 Longwave photovoltaic UV intensity meter (Section 2.8.1.1) was used to ensure no significant discrepancy ($\langle$ 3 μW cm^{-2} over a 40 μW cm^{-2} average lamp intensity) between light intensities used for the various irradiations. The DNA samples were denatured (Section 4.2.1.3) before being used as the antigen in immunoblotting protocols. Table 6.10. lists the irradiation times of the 0.025 mg/ml DNA samples together with the subsequent cut-off dilutions that were determined using immunoblotting with ECL detection. The immunoblotting protocol was repeated a number of times on each irradiated DNA sample. Scanning densitometry was also employed to confirm or negate a "possibly present" antigen/antibody complex.

Time / h	Cut-off dilution
0.5	1:4800
1.5	1:5400
2.5	1:7200
3.8	1:6800
6.0	1:7200

Table 6.10. Irradiation times and cut-off dilutions for 0.025 mg/ml DNA as analysed by immunoblotting with ECL quantitation.

The above results (Table 6.10.) are represented graphically in Figure 6.13. As can be seen, there is an increase in dimer yield with increasing irradiation time, until approximately 2.5 hours is reached. Thereafter a levelling off in dimer concentration is evident in line with the fact that maximum dimer yields are being reached at these irradiation times. Saturation in dimer yields reflects a limitation in substrate (contiguous thymines in the DNA) available for dimerization (i.e. undimerized), rather than

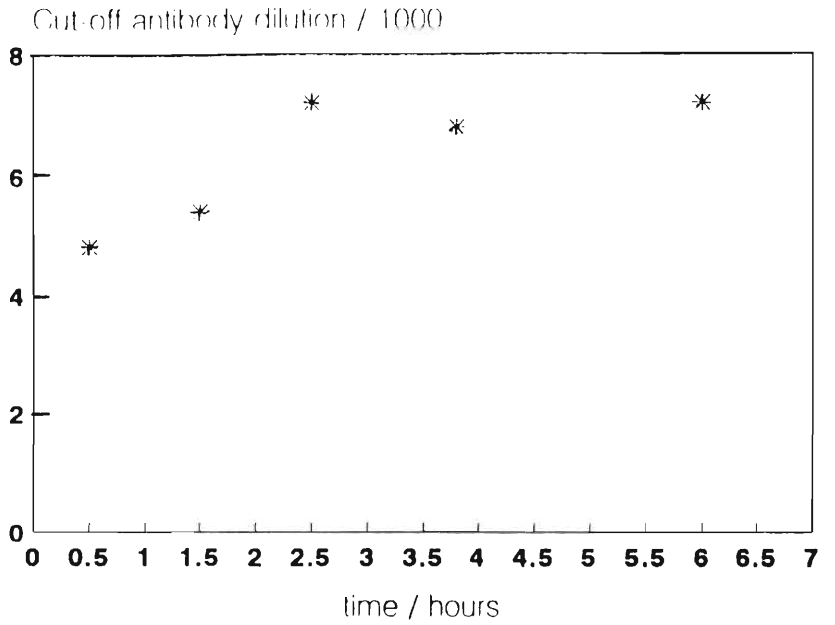


Figure 6.13. Cut-off antibody dilutions as a function of irradiation time, [DNA] = 0.025 mg/ml and [Acp] = 2 x 10^{-2} M.

a limitation in acetophenone available for photosensitized reactions to proceed. The lower cut-off dilution after 3.8 hours represents a 6% decrease in maximum dimer yields which falls within the range of experimental error for a technique where visual observation of the results formed an important part of the analysis.

The slope and intercept from the calibration graph were used to convert the cut-off dilutions (as determined by immunoblotting) to dimer concentrations. Seeing that a linear model ($y = mx + c$) was used, the following equation is applicable:

$$[TT] = \frac{\text{Cut-off dilution - Intercept}}{\text{Slope}}$$

where [TT] refers to the thymine dimer concentration. Table 6.11. gives the calculated dimer yields determined at the 5 irradiation times using the above equation.

Time / h	$[Dimer]_{calc}$ / M	$[Dimer]_{sim}$ / M
0.5	1.125×10^{-7}	5.265×10^{-8}
1.5	1.279×10^{-7}	1.135×10^{-7}
2.5	1.744×10^{-7}	1.455×10^{-7}
3.8	1.640×10^{-7}	1.644×10^{-7}
6.0	1.744×10^{-7}	1.721×10^{-7}

Table 6.11. Experimental dimer yields (from immunoblotting) and simulated dimer yields (outputted from CAKE, see later) for the analysis of 0.025 mg/ml DNA.

The next step is to obtain simulated thymine dimer yields using the proposed mechanism and the computer programme CAKE.

6.6.2 SIMULATION OF THYMINE DIMER YIELDS USING CAKE

CAKE is a computer programme designed for the **C**omputer **A**nalysis of **K**inetic **E**quations[199-201], allowing the solution of a set of differential equations as specified by the user. The equation is set in a Jacobian matrix which is stored within the computer to ensure that a minimum number of operations are performed to solve these non-linear equations. Solution of equations is performed using the Newton-Raphson process with a backward differential formula. CAKE has been used with success in this laboratory[54,110,111,202] and elsewhere[200] in a number of different problems. In particular, Thomas[54] used CAKE in supporting a proposed mechanism for the acetophenone photosensitized irradiation of calf thymus DNA. The mechanism and associated rate constants (see below), initial concentrations of all species and reaction times were supplied to the programme. CAKE used this information to calculate the final thymine dimer concentrations expected. A good agreement between experimental and simulated data was taken by Thomas[54] as support for the validity of the proposed mechanism.

The mechanism as proposed by Thomas[54] is as follows:

gap ---⟩ sap	$k_1 = 1.6 \times 10^{-3}\ s^{-1}$
sap ---⟩ tap	$k_2 = 6.0 \times 10^{10}\ s^{-1}$
tap + gap ---⟩ gap + gap	$k_3 = 1.7 \times 10^{8}\ dm^3mol^{-1}s^{-1}$
tap ---⟩ gap	$k_4 = 5.0 \times 10^{2}\ s^{-1}$
tap + x ---⟩ gap + x	$k_5 = 1.0 \times 10^{8}\ dm^3mol^{-1}s^{-1}$
tap + tt ---⟩ ttt + gap	$k_6 = 4.0 \times 10^{9}\ dm^3mol^{-1}s^{-1}$
tap + tt ---⟩ tt + gap	$k_7 = 1.3 \times 10^{8}\ dm^3mol^{-1}s^{-1}$
ttt ---⟩ dim	$k_8 = 1.1 \times 10^{7}\ s^{-1}$
dim + tap ---⟩ gap + dim	$k_9 = 1.3 \times 10^{8}\ dm^3mol^{-1}s^{-1}$
ttt ---⟩ tt	$k_{10} = 2.0 \times 10^{9}\ s^{-1}$

where gap is the ground state acetophenone, sap is the singlet state acetophenone, tap is the triplet state acetophenone, x is any non-contiguous thymine base, tt is a contiguous thymine pair, ttt is a triplet state thymine pair and dim represents the thymine dimer

The rate constants refer to the following processes:
k_1 is the rate constant obtained from chemical actinometry for the amount of light absorbed by a gap molecule, k_2 is the intersystem rate constant for acetophenone crossing from the singlet to triplet state, k_3 is the the rate constant for acetopheneone self quenching, k_4 is the rate constant for non-radiative decay of triplet acetophenone to the singlet ground state, k_5 is the rate constant for quenching of triplet acetophenone by any non-contiguous thymine bases in the DNA, k_6 is the rate constant for triplet energy transfer from triplet acetophenone to a thymine moeity in a contiguous thymine pair, k_7 is the rate constant for quenching of triplet acetophenone by ground state contiguous thymine pairs, k_8 is the rate constant for dimerization, k_9 is the rate constant for quenching of triplet acetophenone by the thymine dimer photoproduct and k_{10} is the rate constant for decay of triplet thymine to the ground state.

Some of these rate constants were available from literature, whereas the rest were determined from actinometry data or by steady state Stern-Volmer analysis.

The initial concentrations of reactant species were specified as follows: gap = 2.0×10^{-2}, sap = 0.0, tap = 0.0 and ttt = 0.0. The irradiation times of 0.5, 1.5, 2.5, 3.8 and 6 hours were used and the programme run to obtain simulated thymine dimer yields at these various irradiation times. Seeing that experimental dimer yields were obtained at these times using immunoblotting, a direct comparison could be made between the experimental and simulated dimer

yields.

However, the programme also required an initial concentration of contiguous thymine pairs as well as a value for any non-contiguous thymine bases. The former concentration was taken as having the same value as the dimer concentration calculated after 6 hours irradiation[54]. The non-contiguous base concentration was determined as a multiplicative function of 14.65 of the maximum dimer concentration[114]. Table 6.11., lists the simulated dimer yields obtained. These are compared to the experimentally obtained values in Figure 6.14.

If Figure 6.14. is examined, excellent agreement between simulated and experimental dimer yields is achieved at 0.5 and 6.0 hours irradiations while at 3.8 hours irradiation the correlation is fairly good. The only major discrepancies are evident at 1.5 and 2.5 hour irradiations. However, the simulated data line passes between the experimental data points at these irradiation times.

Thus without altering the mechanism proposed for the acetophenone photosensitized irradiation of calf thymus DNA, good correlation between simulated dimer yields (obtained from the mechanism) and those obtained by immunoblotting is evident. It is important to note that this correlation is obtained with dimer yields calculated from simple linear regression of the entire range of calibration points. It therefore appears that the anomaly of non-linearity evident in the calibration graph (Section 6.3.2 and 6.3.3) does not significantly affect these results but does warrant further investigation. By implication, although the volume of antigen delivered to the membrane surface during immunoblotting did cause site restriction when antibody and conjugated secondary antibody were delivered to the membrane surface, this feature did not affect the calibration graph and subsequent simulations

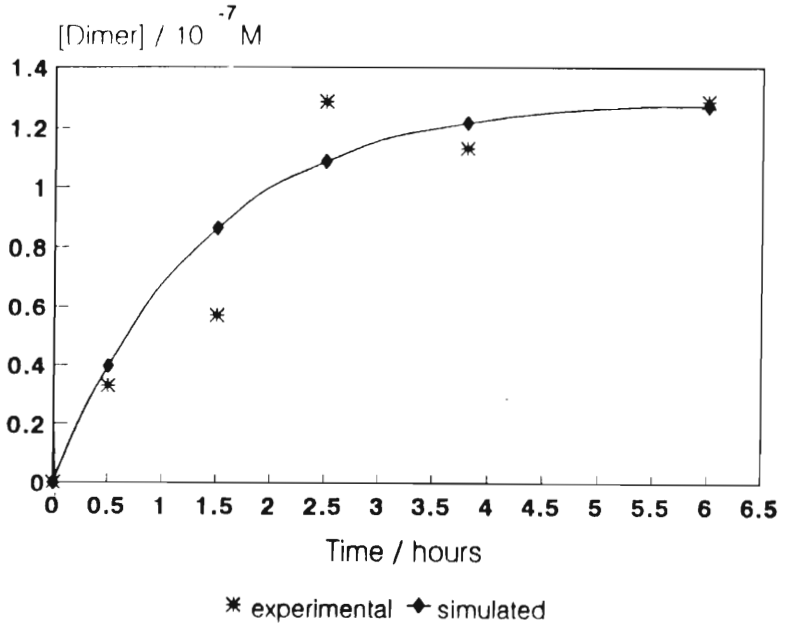


Figure 6.14. Calculated dimer yields (from immunoblotting) and simulated dimer yields (outputted from CAKE) for the analysis of 0.025 mg/ml irradiated DNA.

performed using it. If sample conservation was important, it would obviously have been advantageous to deliver a smaller volume of antigen during immunoblotting. Hawkes et al.[169] note that visible spots can be seen if 0.1 μl antigen is administered to a nitrocellulose membrane in an immunoblotting assay. In contrast, it is not recommended that a volume of greater than 5 μl be administered manually using a micropipette because of the problems of antigen drying on the membrane surface at a slower rate than what the membrane itself dries (Section 6.2.1).

6.7 APPLICATION OF THE MECHANISM AT 0.025 mg/ml DNA

The main aim of the work performed in these laboratories is the elucidation of the kinetics and mechanism of the photosensitized dimerization of pyrimidine bases in DNA in cellular systems. A reductionist approach was adopted investigating first the photosensitized dimerization of thymine, thymidine, TpT[110,111], cytosine and uracil[92] seeing that more information was available in the literature for these simpler systems. Thereafter, the investigation was extended to thymine containing micellar systems[85] and finally to the most complex UV absorbing system, DNA. In this context, the kinetics and mechanism have been elucidated to explain the acetophenone photosensitized dimerization of calf thymus DNA[54] and the para-aminobenzoic acid photosensitized dimerization of pUC19 plasmid DNA[50]. These systems were investigated *in vitro* as a major step to understanding the *in vivo* processes. However, no kinetic study of photosensitized dimerization *in vivo* has been carried out, and it is thus unknown how significantly the kinetics of an *in vivo* system would differ from an *in vitro* one. An *in vivo* mechanism would have to consider the various dimer repair processes occurring simultaneously with dimer formation as well as the scattering and absorption of radiation by various components of the cell, including the outer cell wall and membrane and the nuclear

membrane. Patrick and Gray[203] note that any differences between the *in vitro* and *in vivo* photosensitized irradiation of DNA would be primarily accounted for by the shielding from radiation in the cellular system. In its basic form, the mechanism should thus be the same, apart from taking cognisance of the important repair pathways in the *in vivo* case.

As a step towards confirming or elucidating a mechanism at the cellular level, the technique of immunoblotting was developed to quantitate lesion formation at levels (< 0.05 mg/ml DNA) undetectable by the HPLC in use in these laboratories. Using a calibration graph, cut-off antibody dilutions were converted to thymine dimer concentrations. The kinetics and mechanism as proposed by Thomas[54] for the acetophenone photosensitized irradiation of calf thymus DNA was tested using the experimentally determined thymine dimer concentrations. Without changing the mechanism, or altering any of the kinetic parameters, good correlation between experimental and simulated dimer yields supported the validity of the mechanism at 0.025 mg/ml DNA.

The fact that the data could be applied to the mechanism is an important step forward in moving towards the elucidation of the kinetics and mechanism of photosensitized dimerization of DNA *in vivo*. What is more significant is that it was applied to the mechanism at dimer concentrations which previously were not detectable in these laboratories using HPLC analysis. This of course suggests that the same or similar mechanism is operating at the DNA concentration (0.025 mg/ml) four times lower than the concentration at which at which the mechanism was previously tested. In these studies the acetophenone concentration was kept constant at 2×10^{-2} M. A decrease in DNA concentration implies a higher effective acetophenone concentration available for photodimerization at wavelengths greater than 300 nm. However, saturation limits

for sensitized dimerization must be accounted for primarily because of nucleation phenomena sterically reducing thymine residues available for dimerization[53]. As a consequence it is postulated that of the acetophenone which is photoexcited, only a certain proportion may induce dimerization, the remainder of which must decay to the ground state via non-radiative decay or fluorescence as proposed in the mechanism of Thomas. In extending these studies the mechanism will need to be tested at sequentially lower DNA concentrations.

Evidence for the same mechanism operating over a range of DNA systems is provided by the work performed by Aliwell[50] in these laboratories. Using the triplet photosensitizer para-aminobenzioc acid (PABA, found in many sunscreen preparations as an active UV absorber), the photodimerization of aqueous free thymine, thymidylyl-3′,5′-thymidine (TpT) and pUC19 plasmid DNA were investigated. HPLC analysis was used to quantitate thymine dimer yields and mechanistic and kinetic data determined. In its bare form, the proposed mechanism accounted for the excitation of PABA to triplet PABA, the transfer of this energy to free thymine molecules or contiguous thymine pairs (as in TpT and pUC19), the quenching of triplet PABA by free thymine molecules, TpT or non-contiguous thymine moeities in DNA and finally dimerization of contiguous thymine pairs. The significant feature of this mechanism is that it proposed to be operational within all these thymine containing systems, each one representing a gradation from the simplest (free thymine) to the most complex (DNA) system. This gives support to the work presented here which showed that the same mechanism was valid over a range of DNA concentrations.

6.8 SUGGESTIONS FOR FUTURE WORK

This thesis has described an immunoblotting protocol with enhanced chemiluminescent detection for the quantitation of cis-syn thymine dimers. The protocol was successful in quantitating dimers at levels above the detection limit of the HPLC (currently used in these laboratories for dimer quantitation). It was also used successfully in dimer quantitation at levels undetectable by the HPLC and to this end the immuno technique was between 4000 to 8000 more sensitive than the chromatographic technique.

With the planned extension of this work to thymine dimer detection at cellular levels, it is hoped that immunoblotting with enhanced chemiluminescent quantitation will provide the necessary sensitivity required.

In these laboratories quantitation of thymine dimers in cellular systems was initiated by irradiation of *E. coli* bacteria in the presence of acetophenone[51]. Although problems were experienced in absorption of acetophenone by the bacteria, this irradiation substrate does find application in the work presented here. The DNA of irradiated *E. coli* can be extracted (lysosyme in Tris to lyse the cell walls, followed by addition of Triton X-100 to sever the cell membrane, thereby releasing the DNA and other cell components into solution) and purified (phenol-chloroform for protein removal folowed by RNAse treatment for removal of RNA components). The DNA can then be used as the antigen in an immunoblotting protocol. It will also be necessary to raise antibodies to the extracted DNA, taking care to produce as uncontaminated a DNA sample as possible during the extraction and purification procedures.

An important advantage of the immunoblotting technique is the sensitivity it confers to the system under investigation. Unlike the attempts made by Bolton[51] to

irradiate litres of cultured *E. Coli* cells in the presence of acetophenone, immunoblotting analysis of irradiated and extracted DNA would require a much smaller volume or a lower concentration of thymine dimer for analysis, so smaller volumes of *E. coli* cells need to be irradiated. To this end a calculation was performed to estimate the quantity of cells that need to be irradiated for such a purpose.

A single *E. coli* cell contains approximately 5×10^{-15} g of DNA[204]. Using the molecular structure of an oligonucleotide containing all four nitrogenous bases (Figure 1.2.), it was calculated that 58.934% of the structure (by mass) consists of the phosphate-deoxyribose backbone. Thus in the single *E. coli* cell, 41.066% (i.e. 2.0532×10^{-15} g) of the DNA is comprised of the nitrogenous bases. If 25.4% of the bases are thymine (a mass percentage is assumed, since no mention was made by Josse et al.[205] as to a mass or molar percentage), then 5.2151×10^{-16} g of thymine is present in a single cell. For dimerization to occur, the thymine moeities must be adjacent to one another. In this context, the nearest neighbour frequency for thymine is quoted to be 0.076[205]. Thus 3.9634×10^{-17} g of the DNA is present as dimerizable thymine residues. Under acetophenone photosensitizing conditions, approximately 95% of the thymine bases will dimerize to give thymine dimers[26], thus it is possible that 3.7652×10^{-17} g of the DNA will exist as thymine dimers after irradiation. Taking the molar mass of the thymine dimer to be 252 g/mol, 1.4941×10^{-19} mol of dimer could be present.

The sensitivity of immunoblotting as determined in this work is 2.5×10^{-10} M dimer (Section 6.5) detected in a 5 μl aliquot of irradiated DNA blotted onto the membrane surface during an immunoblotting protocol. In this volume of DNA, 1.25×10^{-10} mol thymine dimer was thus detected. A single protocol may require approximately 20 aliquots to be

blotted. Thus 2.50×10^{-14} mol thymine dimer is the minimum number of moles of thymine that needs to be extracted from the cellular substrate. If a single cell contains 1.4941×10^{-19} mol thymine dimer (as calculated above), then a minimum of approximately 167000 *E. coli* cells must be irradiated and the DNA extracted.

It must be added that this figure is merely an estimate as to the number of cells needed to run an immunoblotting protocol. In this regard, the DNA content of the cell is merely an approximation, as the chromosome number of the *E. coli* may vary according to whether the cell is in a replication cycle or not. If such an irradiation and extraction was to be performed, it would also be necessary to consider the efficiency of the extraction procedure itself, as well as the purity of the DNA preparation.

However, in its bare form, this calculation does provide a starting point in applying the immunoblotting protocol to the detection of dimers in cellular systems. When the work proves successful in this application, it can then be extended to other irradiation substrates (e.g. human skin cells) and kinetic mechanisms (proposed to explain the acetophenone photosensitized irradiation of calf thymus DNA[54]) tested in all cases. If the mechanism is found to apply (with or without modification) then the work presented in these laboratories will provide important evidence in elucidating the initiation of neoplastic events at cellular levels.

6.9 CONCLUSION

This thesis presents an immunological approach for the detection of cis-syn thymine dimers in UV-irradiated calf thymus DNA. The UV-DNA antigen was injected into laboratory rabbits and the serum (containing the UV-DNA antibodies) obtained by bleeding the ear vein.

The crude serum was purified using the technique of ammonium sulphate precipitation of proteins as well as by the more sophisticated technique of affinity chromatography. The latter involved the selective binding of the IgG component of the serum to Protein A and the procedure was developed and optimized on a Waters Delta Prep 4000 HPLC system.

The immunoassay technique of immunoblotting was chosen to immobilize the antigen, antibody and a secondary antibody (conjugated to the enzyme, horseradish peroxidase) on a commercially available membrane system. The technique of enhanced chemiluminescence was then chosen to detect and quantitate the immune complexes. This involved the addition of luminol to the membrane bound complexes, and resulted in the horseradish peroxidase catalysing the conversion of luminol and one of the products (light of 425 nm to 430 nm) impinging on a luminescence film. The film was then developed and printed using standard photographic techniques.

Development of a protocol for the detection and quantitation of thymine dimer involved the selection of reagents and equipment from a wide commercial range, and optimization of the reagent concentrations and incubation times so as to yield the clearest signal on the luminescence film. To this end, immunoblotting with ECL detection allowed the quantitation of cis-syn dimer lesions at 2.5×10^{-10} M - the technique being between 4000 to 8000

times more sensitive than HPLC currently used in this laboratory.

Characterization of the antibody population indicated that 40% of the antibody was directed against the thymine dimer, with the remaining percentage being produced in response to the deoxyribose-phosphate backbone, purine and pyrimidine bases of the DNA and to a small percentage of acetophenone.

A calibration graph of dimer concentration versus cut-off antibody dilutions was constructed. Non-linearity of the graph was explained in terms of site restriction and stereochemical constraints of the antibodies coming into contact with the antigen on the membrane surface.

Using immunoblotting with ECL detection and quantitation, the calibration graph was used to calculate thymine dimer yields at 0.025 mg/ml DNA concentration - at this concentration thymine dimers are undetectable by HPLC techniques. The previously determined kinetic mechanism for the acetophenone photosensitized dimerization of calf thymus DNA ([DNA] > 0.1 mg/ml) was used to model dimer formation at this lower DNA concentration using the computer programme CAKE. A good agreement between experimental and simulated data was taken as support for the applicability of this mechanism to the irradiated 0.025 mg/ml DNA solution.

The implications for the same kinetic mechanism operating at a range of DNA concentrations (0.025 mg/ml to 10.0 mg/ml) are manifold. The same mechanism may well be operational at DNA concentrations (μg/ml) present in cellular systems and it is hoped that the work presented in this thesis will provide a stepping stone in elucidating cis-syn dimer (and other photoproducts) yields as well as the kinetic mechanisms for their formation in UV-irradiated cellular systems. In this manner, the molecular basis

underlying UV-induced carcinogenesis may well be better understood.

REFERENCES

1. Watson J.D. and Crick F.H.C., Nature **171** (1953) 737.
2. Patrick M.H., Photochemistry and Photobiology of Nucleic Acids, Vol 2, Wang S.Y. (Ed.), (1976), Academic Press, New York, pp 1-34.
3. Stollar B.D., Arth. Rheum. **24** (1981) 1010.
4. Conn E.C. and Stumpf P.K., Outlines of Biochemistry, 4th Edition, (1976), John Wiley and Sons, New York, p. 113.
5. Phillips D.H., The Molecular Basis of Cancer, Farmer P.B. and Walker J.M. (Eds.), (1985), Croom Helm, London, pp 133-181.
6. Walker I. and Conklin J.J., Military Radiobiology, Academic Press, (1987), Chapter 1.
7. Doniger J., Jacobson E.D., Krell K. and Di Paolo D.A., Proc. Nat. Acad. Sci. **78** (1981) 2378.
8. Sutherland B.M., Delihas N.C., Oliver R.P. and Sutherland J.C., Cancer Res. **41** (1981) 2211.
9. Cleaver J.E. and Bootsma D., Annu. Rev. Genet. **9** (1975) 19.
10. Hart R.W., Setlow R.B. and Woodhead A.D., Proc. Nat. Acad. Sci. **75** (1977) 5574.
11. Setlow R.B., Woodhead A.D. and Grist E., Proc. Nat. Acad. Sci. **86** (1989) 8926.
12. Ley R.D., Applegate, L.A., Fry R.J.M. and Stuart T.D., Photochem. Photobiol. **47** (1988) 45S (Abstr.).
13. Raghunathan G., Kieber-Emmons T., Rein R. and Alderfer J., J. Mol. Str. Dyn. **7** (1990) 899.
14. Peak M.J., Peak J.G., Moehring M.P. and Webb R.B., Photochem. Photobiol. **40** (1984) 613.
15. Rosenstein B.S. and Ducore J.M., Photochem. Photobiol. **38** (1983) 51.
16. Jones C.A., Huberman E., Cunningham M.L. and Peak J.G., Radiat. Res. **110** (1987) 244.

17. Setlow R.B. and Ahmed F.E., Carcinogenesis: Fundamental Mechanisms and Environmental Effects, Pulman B., Tso P. and Gelboin H., (Eds.), (1980), D. Riedel Publishing Company, New York, p. 435.
18. Summers B. and Summers R.S., S.A. Pharmaceutical Journal August (1991) 231.
19. Fourtanier A., Labat-Robert J., Berrebi C. and Kern P., J. Invest. Dermatol. **92** (1989) 429.
20. Roza L., Baan R.A., Van der Leun J., Kligman L. and Young A.R., Photochem. Photobiol. B. Biol. **3** (1989) 281.
21. Kaidby K.H. and Kligman A.M., J. Invest. Dermatol. 78 (1982) 371.
22. Beukers R. and Berends W., Biochim. Biophys. Acta **41** (1960) 550.
23. Weinblum D., Biochem. Biophys. Res. Commun. **27** (1967) 384.
24. Wacker A., Dellweg H. and Weinblum D., Naturwissenschaften **47** (1960) 477.
25. Wang. S.Y. and Varghese A.J., Biochem. Biophys. Res. Commun. **29** (1967) 543.
26. Regan J.D., Carrier W.L. and Francis A.A., Photomed. Photobiol. **6** (1984) 1.
27. Patrick M.H., Photochem. Photobiol. **25** (1977) 357.
28. Brash D.E. and Haseltine W.A., Nature **285** (1982) 189.
29. Eker A.P.M., Molecular Models of Responsiveness, Montagnali G. and Erlanger B.F., (Eds.), (1983), Plenum Press, New York, p. 109 to 132.
30. Ciarrocchi G. and Sutherland B.M., Photochem. Photobiol. **38** (1983) 259.
31. Pearlman D.A., Holbrook, S.R., Pirkle D.H. and Kim S.H., Science **227** (1984) 1304.
32. Setlow J.K., Boling M.E. and Bollum F.J., Proc. Nat. Acad. Sci. **53** (1965) 1430.
33. Setlow J.K. and Setlow R.B., Nature **197** (1963) 560.
34. Rupert C.S., J. Gen. Physiol. **45** (1962) 725.
35. Moan J. and Peak M.J., Photochem. Photobiol. B. Biol.

4 (1989) 21.

36. Sancar A. and Rupp W.D., Cell **33** (1983) 249.
37. Mayne L.V. and Lehmann A.R., Cancer Res. **42** (1989) 1473.
38. Zajdela F. and Latarjet R., Natl. Cancer Inst. Monogr. **50** (1978) 133.
39. Roza L., Van Der Schans G.P. and Lohman P.H.M., Photochem. Photobiol. **41** (1985) 437.
40. McGhie J.B., Wold E., Pettersen E.O. and Moan J., Radiat. Res. **96** (1983) 31.
41. Hanawalt P.C. and Sarasin A., Trends Genet. **2** (1986) 124.
42. Lehmann A.R. and Norris P.G., Carcinogenesis **10** (1989) 1353.
43. Westerveld A., Hoeijmakers J.H.M., Odjik H., Pastink A., Wood R.D. and Bootsma D., Nature **310** (1984) 425.
44. Brash D.E., Photochem. Photobiol. **48** (1988) 59.
45. Schaaper R.M. and Glickman B.W., Mol. Gen. Genet. **185** (1982) 407.
46. Von Welucki I., Matthaus H. and Krauch C.H., Photochem. Photobiol. **6** (1967) 497.
47. Greenstock C.L. and Johns H.E., Biochem. Biophys. Res. Commun. **30** (1968) 21.
48. Lamola A.A., Pure and App. Chem. **24** (1970) 599.
49. Charlier M. and Helene C., Photochem. Photobiol. **6** (1967) 501.
50. Aliwell S.R., M.Sc. Thesis (1992), University of Natal, Durban, South Africa.
51. Bolton K., M. Sc. Thesis (1992), University of Natal, Durban, South Africa.
52. Henderson B.W. and Dougherty T.J., Photochem. Photobiol. **55** (1992) 145.
53. Texter J., Biopolymers **30** (1990) 797.
54. Thomas R.C., M.Sc. Thesis (1990), University of Natal, Durban, South Africa.
55. Patrick M.H. and Rahn R.O., Photochemistry and Photobiology of Nucleic Acids, Vol 2, Wang S.Y.

(Ed.), (1976), Academic Press, New York, pp 35-49.

56. Sutherland B.M. and Sutherland J.C., Biophys. J. **9** (1969) 1329.
57. Varghese A.J. and Patrick M.H., Nature **223** (1969) 299.
58. Weinblum D. and Johns H.E., Biochim. Biophys. Acta **114** (1966) 450.
59. Mitchell D.L. and Nairn R.S., Photochem. Photobiol. **49** (1989) 805.
60. Von Zeeland A.A., Smith C.A. and Hanawalt P.C., Mutat. Res. **82** (1981) 173.
61. Frenkel K., Goldstein M.S. and Teebor G.W., Biochemistry **20** (1981) 7566.
62. Olsen W.H. and Storeng R., Pharmacol. Toxicol. **61** (1987) 203.
63. Weir D.M., Immunology: An Outline for Students of Medicine and Biology, 5th Edition, (1983), Longman Group Ltd, pp 57-59.
64. Braun W., Whallon J. and Phillips J., Nature **180** (1957) 1356.
65. Phillips J., Braun W. and Plescia O.J., J. Am. Chem. Soc. **80** (1958) 2710.
66. Plescia O.J., Braun W. and Palczuk N.C., Proc. Nat. Acad. Sci. **52** (1964) 279.
67. Plescia O.J. Palczuk N.C., Braun W. and Cora-Figueroa E., Science **148** (1965) 1102.
68. Seaman E., Levine L. and Van Vunakis H., Biochem. **4** (1965) 2091.
69. Plescia O.J., Palczuk N.C., Cora-Figueroa E., Mukherjee A. and Braun W., Proc. Nat. Acad. Sci. **54** (1965) 1281.
70. Van Vunakis H., Seaman E., Kahan L., Kappler J.W. and Levine L., Biochem. **5** (1966) 3986.
71. Levine L., Seaman E., Hammerschlag E. and Van Vunakis H., Science **153** (1966) 1666.
72. Cornelis J.J., Rommelaere J., Urbain J. and Errera M., Photochem. Photobiol. **26** (1977) 241.
73. Ley R.D., Cancer Res. **43** (1983) 41.

74. Seaman E., Van Vunakis H., Levine L., J. Biol.Chem. **247** (1972) 5709.
75. Natali P.G. and Tan E.M., Radiat. Res. **46** (1971) 506.
76. Strickland P.T. and Boyle J.M., Photochem. Photobiol. **34** (1981) 595.
77. Lucas C.J., Exp. Cell. Res. **74** (1972) 480.
78. Seaman E., Levine L. and Van Vunakis H., Nucleic Acids in Immunology, Plescia O.J. and Braun W., (Eds.), (1972) Springer-Verlag, Berlin and New York, p. 157.
79. Slor H., DNA Repair and Late Effects, Altmann H., Riklis E., and Slor H, (Eds.), (1980) Nuclear Research Centre, Negev, Israel, p. 103.
80. Mori T., Nakane M., Hattori T., Matsunaga T., Ihara M. and Nikaido O., Photochem. Photobiol. **54** (1991) 225.
81. Wazizaka A. and Okuhara E., J. Biochem. **86** (1979) 1469.
82. Leipold B., Remy W. and Adelmann-Grill B., J. Immuno. Methods **60** (1983) 69.
83. Southern E.M., J. Mol. Biol. **98** (1975) 503.
84. Nehls P., Adamkiewicz J. and Rajewsky M.F., J. Cancer Res. Clin. Oncol. **108** (1984) 23.
85. Malone M.E., M.Sc. Thesis (1990), University of Natal, Durban, South Africa.
86. Wang S.Y., Nature **200** (1968) 879.
87. Fisher G.J. and Johns H.E., Photochem. Photobiol. **11** (1970) 429.
88. Fuchtbauer W. and Masur P., Photochem. Photobiol. **5** (1966) 323.
89. Marmur J. and Doty P., Nature **183** (1959) 428
90. Mahler H.R. and Cordes E.H., Biological Chemistry, (1966), Harper and Row Publishers Incorporated, New York, p. 140.
91. Hurley I., Osei-Gyimah P., Archer S., Scholes C.P. and Lerman L.S., Biochem. **21** (1982) 4999.
92. De Bruyn W., M. Sc. Thesis (1990), University of Natal, Durban, South Africa.
93. Lamola A.A. and Yamane T., Proc. Nat. Acad. Sci. **58** (1967) 443.

94. Rahn R.O. and Stafford R.S., Nature **248** (1974) 52.
95. Rahn R.O. and Patrick M., Photochemistry and Photobiology of Nucleic Acids, Vol 2, Wang S.Y. (Ed.), (1976), Academic Press, New York, pp 123-129 (Rahn R.O., unpublished data).
96. Smith K.C., Photochem. Photobiol. **2** (1960) 503.
97. Cook K.H. and Friedberg E.C., Anal. Biochem. **73** (1976) 411.
98. Wacker A. Prog. Nucl. Acid Res. **1** (1963) 369.
99. Lakings D.B. and Gerke C.W., J. Chromatog. **62** (1971) 347.
100. McBurney A. and Gibson T., Clin. Chem. Acta. **102** (1980) 19.
101. Taylor G., Dady P.J. and Harrap K.R., J. Chromatog. **1983** (1980) 421.
102. Simmons R.J. and Harkness R.A., J. Chromat. **226** (1981) 369.
103. Salas C.E. and Sellinger O.Z., J. Chromatog. **133** (1977) 231.
104. Rustum Y.M., Anal. Biochem. **90** (1978) 289.
105. Ramos D.L. and Schoffstall A.M., J. Chromatog. **261** (1980) 83.
106. Wakizaka A., Kurosaka K. and Okuhara E., J. Chromatog. **162** (1979) 319.
107. Cadet J., Voituriez L., Hahn B.S. and Wang S.Y., J. Chromatog. **195** (1980) 139.
108. Cadet J., Berger D. and Voituriez L., J. Chromatog. **238** (1982) 488.
109. Niggli H., Cosmet. Derm. Vol 1, Morganti P. and Montagna W. (Eds), International Edition, 335.
110. Foster S.J., M.Sc. Thesis, (1989), University of Natal, Durban, South Africa.
111. Gravett D.G., M.Sc. Thesis, (1990), University of Natal, Durban, South Africa.
112. Voet D., Gratzer B., Cox R.A. and Doty P., Biopolymers **1** (1963) 193.
113. Zakaria M. and Brown R.P., J. Chromatog. **226** (1981)

267.

114. Clemmett S.J., M.Sc. Thesis, University of Natal, Durban, South Africa, in preparation.
115. Rosen F.S., Steiner L.A. and Unanue E.R., Macmillan Dictionary of Immunology, (1989), Macmillan Press, London, p. 202.
116. Stollar B.D., The Antigens, Vol 1, Sela M. (Ed) (1973) Academic Press, New York, pp 1 to 85.
117. Stollar B.D., Crit. Rev. Biochem. **3** (1975) 45.
118. Stollar B.D. and Grossman L., J. Mol. Biol. **4** (1962) 13.
119. Plummer D.T., An Introduction to Practical Biochemistry, 2nd edition, (1978) McGraw-Hill, London, p. 238.
120. Mahler H.R. and Cordes E.H., Biological Chemistry, (1966), Harper and Row Publishers Incorporated, New York, pp 165-174.
121. Doty P., Marmur J., Eigner J. and Schildkraut C., Biochem. **46** (1960) 461.
122. Charlier M. and Helene C., Acad. Sci., Ser. D. **272** (1971) 743.
123. Zierenberg B.E., Kramer D., Geisert M.G. and Kirste R.G., Photchem. Photobiol. **14** (1971) 515.
124. Wani A.A., Gibson D'Ambrosio R.E. and D'Ambrosio S.M., Photochem. Photobiol. **40** (1984) 465.
125. Halloran M.J. and Parker C.W., J.Immuno. **96** (1966) 373.
126. Maurer P.H., J. Exptl. Med. **121** (1965) 339.
127. Seaman E., Levine L. and Van Vunakis H., Biochem. **5** (1966) 1216.
128. Sela M., Science **166** (1969) 1365.
129. Sueoka M. and Cheng T., J. Mol. Biol. **4** (1962) 161.
130. Freund J. and McDermott K., Proc. Soc. Exp. Biol. Med. **49** (1942) 548.
131. Hurn A. L., Chantler S.M., Methods in Enzymology, Vol 70A, Colowick S.P. and Kaplan N.O. (Eds), (1980), Academic Press, New York, pp 109-142.

132. Van Vunakis H., Seaman E. and Levine L., Nucleic Acids In Immunology, Plescia O.J. and Braun W. (Eds), (1968) Springer-Verlag, New York, pp 236-237, (Plescia and Braun, unpublished data, 1966).

133. Mahler H.R. and Cordes E.H., Biological Chemistry, (1966), Harper and Row Publishers Incorporated, New York, pp 8-32.

134. Plummer D.T., An Introduction to Practical Biochemistry, (1978), 2nd edition, McGraw-Hill, London, p. 65.

135. Phillips R.G. and Kalthoff K., Photochem. Photobiol. **48** (1988) 77.

136. Szewczuk A. and Prusak E., Affinity Chromatography: A Practical Approach, Dean P.G.D., Johnson W.S. and Middle F.A. (Eds), (1985), IRL Press, Oxford, pp 118 -119.

137. Cornelis J.J. and Errera M., DNA Repair: A Laboratory Manual of Research Proceedings, Vol 1A, Friedberg E.C. and Hanawalt P.C. (Eds), (1981), Marcel Decker, New York, pp 31-34.

138. Mahler H.R. and Cordes E.H. Biological Chemistry, (1966), Harper and Row Publishers Incorporated, New York, pp 63-69.

139. Chard T., Methods In Enzymology, Vol 70, Van Vunakis H. and Langone J.J. (Eds), (1980), Academic Press, New York, pp 280-291.

140. Nowotny A., Basic Exercises in Immunochemistry: A Laboratory Manual, (1979), Springer-Verlag, Berlin, pp 1-3.

141. Dawson R.M., Elliot D.C., Elliot W.H. and Jones K.M. Data for Biochemical Research, 3rd edition, (1986), Oxford University Press, Oxford, pp 537-539.

142. Regnier F.E., Liquid Chromatography Gas Chromatography **5** (1987) 962.

143. Goheen S.C. and Matson R.S., J.Chromatog. **326** (1985) 235.

144. Stringham R. and Regnier F.E., J. Chromatog. in press

from reference 142.

145. Clezardin P., Mc Gregor J.L., Manach M., Boukerche H. and Dechavanne M., J.Chromatog. **319** (1985) 67.

146. Pavlu B., Johannson U., Nyhlen C. and Wichman A., J. Chromatog. **359** (1986) 449.

147. Smith G.J., McFarland R.D., Reisner H.M. and Hudson G.S., Anal. Biochem. **141** (1984) 432.

148. Juarez-Salinas H., Ott G.S., Chen J.C., Brooks T.L. and Stanker H.L., Methods in Enzymology, Vol 121, (1986), Academic Press, New York, pp 615-622.

149. Kronvall G., Grey H.M. and Williams R.C., J.Immuno. **105** (1970) 1116.

150 Dawson R.M., Elliot D.C., Elliot W.H. and Jones K.M., Data for Biochemical Research, 3rd edition, (1986), Oxford Press, Oxford, p. 527.

151. Forsgren A. and Sjoquist J., J. Immuno. **99** (1967) 19.

152. Kronvall G., Quie P.G. and Williams R.C., J. Immuno. **104** (1970) 273.

153. Langone J.J., J. Immuno. Methods **24** (1978) 269.

154. Jangone J.J., Boyle M.D.P. and Borsus T., J. Immuno. Methods **18** (1977) 281.

155. Mota G., Ghetie V. and Sjoquist J., Immunochem. **15** (1978) 639.

156. Lind I. and Mansa B., Acta. Path. Microbiol. Scand. **73** (1968) 637.

157. Gemski M.J., Doctor B.P., Gentry M.K., Pluskal M.G. and Strickler M.P., Biotechniques **3** (1985) 378.

158. Ey P.L., Prowse S.J. and Jenkin C.R., Immunochem. **15** (1978) 429.

159. Tan E.M. and Stoughton R.B., Proc. Nat. Acad. Sci. **62** (1969) 708.

160. Strickland P.T., Enviro. Mutagenesis **7** (1985) 599.

161. Plaza S., Boullard A., Pele D., Cornelis J.J., Rommelaere J., Giacomini P.U. and Prunieras M., Photochem. Photobiol. **53** (1991) 217.

162. Wani A.A., D' Ambrosio S.M. and Alvi N.K., Photochem. Photobiol. **46** (1987) 477.

163. Gershoni J.M. and Palade G.E., Anal. Biochem. **131** (1983) 1.

164. Alwine J.C., Kemp D.J., Reiser J., Renart J., Stark G.R. and Wahl G.M., Methods In Enzymology, Vol 68, Wu R. (Ed) Academic Press, New York, p. 220 to 242.

165. Reiser J. and Wardale J., Eur. J. Biochem. **114** (1981) 569.

166. Fonong T., Evans S.M. and Homberger H.A., Clin. Chem. **36** (1990) 2053.

167. Thomas N., Jones C.N. and Thomas P.L. Anal. Biochem. **170** (1988) 393.

168. Mizuno T., Matsunaga T., Ihara M. and Nikaido O., Mutat. Res. **254** (1991) 175.

169. Hawkes R., Niday E. and Gordon J., Anal.Biochem. **119** (1982) 142.

170. Nakane P.K. and Kawaoi A., J. Histochem. Cytochem. **22** (1974) 1084.

171. Nakane P.K. and Pierce G.B. J. Histochem. Cytochem. **14** (1966) 929.

172. Mullerman H.G., Ter Hart H.G. and Dijk W.V., Anal. Biochem. **120** (1982) 46.

173. Peng C.T. Liquid Scintillation, Science and Technology, Noujaim A.S., C. Ediss and Weibe L.I. (Eds.) Academic Press, New York, pp 313-330.

174. White E.H. and Bursey M.M., J. Am. Chem. Soc. **86** (1964) 941.

175. Isacsson U. and Wettermark G., Anal. Chim. Acta. **68** (1974) 339.

176. Whitehead T.P., Kricka L.J., Carter T.J.N. and Thorpe G.H.G., Clin. Chem. **25** (1979) 1531.

177. Auses J.P. Cook S.L. and Maloy S.T., Anal. Chem. **47** (1975) 244.

178. Gorus F. and Schram E., Clin Chem. **25** (1979) 512.

179. Williams D.C. and Seitz W.R., Anal. Chem. **48** (1976) 1478.

180. Halmann M., Velan B. and Sery T., Appl. Environ. Microbiol., **34** (1977) 473.

181. Schroeder H.R., Hines C.M., Osborn D.D., Moore R.P., Hurtle R.L., Wogoman F.F., Rogers R.W. and Vogelhut P.O., Clin. Chem. **27** (1981) 1378.

182. Puget K., Michelson A.M. and Avrameas S., Anal. Biochem. **79** (1979) 447.

183. Williams D.C., Huff G.G. and Seitz W.R. Anal. Chem. **48** (1976) 1003.

184. Whitehead T.P., Thorpe G.H.G., Carter T.J.N., Groucott C. and Kricka L.J., Nature **305** (1983) 158.

185. Thorpe G.H.G., Whitehead T.P., Penn R.and Kricka L.J., Clin. Chem. **30** (1984) 806.

186. Kendall F.E., J. Clin. Invest. **16** (1937) 921.

187. Eggset G., Krokan H. and Volden G., Photochem. Photophys. **10** (1986) 181.

188. Nussenweig R.S., Merryman C. and Benacerraf B., J. Exp. Med. **120** (1964) 315.

189. Warner C. and Schumaker V., Biochem. **9** (1970) 3040.

190. Munns T.W. and Liszewski M.K. Progress in Nucleic Acid Research and Molecular Biology, Vol 24, Cohn W.E. (Ed), Academic Press, London, p. 133.

191. Olsson T., Brunius G., Carlsson H.E. and Thore A., Clin. Chem. **25** (1979) 1531.

192. Posch N.A., Wells A.F., Tenoso H.J., Detection of Hepatitis in Blood, National Heart and Lung Institute, NIH, Bethesda (1973).

193. Mitchell D.L., Clarkson J.M., Biochim. Biophys. Acta, **655** (1981) 54.

194. Mori T., Nakane M., Hattori T., Matsunaga T. and Nikaido O., Photomed. Photobiol. **12** (1990) 203.

195. Cornelis J.J., Nucleic Acid Res. **5** (1978) 4273.

196. Klocker H., Auer B., Burtcher H.J. Hofmann J., Kaufmann M. and Sweiger M., Mol. Gen. Genet. **186** (1982) 475.

197. Matsunaga T., Mori T. and Nikaido O., Mutat. Res. **235** (1990) 187.

198. Strickland P.T. and Boyle J.M., J. Immuno. Methods **41** (1981) 115.

199. Ridler P.F. and Sheppard J.G., S. Afr. J. Chem. **30** (1979) 49.
200. Ridler G.M., Ridler P.F. and Sheppard J.G., S.Afr. J. Chem. **30** (1977) 55.
201. Ridler P.F., J. Phys. Chem. **81** (1977) 2419.
202. Kilfoil V. and Salter L.F., Int. J. Chem. Kin. **20** (1988) 645.
203. Patrick M.H. and Gray D.M., Photochem. Photobiol. **24** (1976) 507.
204. B. Huckett, personal communication.
205. Josse J., Kaiser A.D. and Kornberg A., J. Biol. Chem. **236** (1961) 864.

APPENDIX A

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start photosensitized irradiation of DNA
equations
gap -> sap >1.6e-3
sap -> tap >6.0e10
tap + gap -> gap + gap >1.7e8
tap -> gap >5.0e2
tap + x -> gap + x >1.0e8
tap + tt -> ttt + gap >4.0e9
tap + tt -> tt + gap >1.3e8
ttt -> dim >1.1e7
dim + tap -> gap + dim >1.3e8
ttt -> tt >2.0e9
initially
gap = 2.0e-2
tap = 0.0
sap = 0.0
ttt = 0.0
dim = 0.0
tt = 1.2887e-6
x = 1.8879e-5
display final
time final = 1.8e3
alter
time final = 5.4e3
alter
time final = 9.0e3
alter
time final = 1.38e4
alter
time final = 2.16e4
finish
```

where gap = ground state acetophenone
sap = singlet state acetophenone
tap = triplet state acetophenone
x = any non-contiguous thymine base
tt = contiguous thymine pair
ttt = triplet contiguous thymine pair
dim = dimer
The 'alter' step allows for a new set of initial concentrations to be run.

APPENDIX B

The following papers and posters were presented at conferences during and after the course of this work:

FIFTH ANNUAL BIOCHEMISTRY SYMPOSIUM (NATAL REGION)
Pietermaritzburg, South Africa, 16 October 1991

"Immunological techniques for the quantitation of thymine dimer in DNA"

A. Kriste, L.F. Salter and B.S. Martincigh.

SOUTH AFRICAN CHEMICAL INSTITUTE
NATIONAL PHYSICAL CHEMISTRY CONVENTION
Durban, South Africa, 29-31 January 1992

"Spectroscopic and chromatographic techniques for the quantitation of thymine dimer generated in ultraviolet-irradiated DNA"

A. Kriste, L.F. Salter and B.S. Martincigh.

7TH BIENNIAL CONGRESS OF THE SOUTH AFRICAN IMMUNOLOGY SOCIETY
Cape Town, South Africa, 15-19 March 1992

"The application of immunoassay techniques to the quantitation of thymine dimer generated in ultraviolet-irradiated DNA"

A. Kriste, L.F. Salter and B.S. Martincigh.

11th INTERNATIONAL CONGRESS ON PHOTOBIOLOGY
Kyoto, Japan, 7-12 September 1992

" Immunoassay detection of ultraviolet-irradiation damage in DNA"

A. Kriste, L.F. Salter and B.S. Martincigh.