A STUDY OF THE DIRECT EFFECTS OF
IONISING AND FAR ULTRAVIOLET
RADIATION ON NUCLEIC ACIDS

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by

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To my parents.
SUMMARY

This thesis reports the results of a study of the direct effects of gamma and far UV radiation on nucleic acid model systems. For the gamma study, frozen aqueous solutions of 2'-deoxyribonucleosides were chosen as the model systems which best mimic possible radiation chemical events via the direct effects occurring in DNA in vivo.

The low yields and high complexity of the mixtures of degradation products afforded by the direct effects required the development of an efficient analytical procedure. This methodology, once perfected for the most complicated system, that of thymidine, was readily applicable to the study of the direct effects of gamma radiation on other nucleosides and to the study of the direct effects of far UV radiation on thymidine.

In Chapter I, we report and discuss the results of the study of the direct effects of gamma radiation on thymidine including the isolation and identification of the chemical modifications induced, and describe experiments designed to probe the mechanisms involved in their formation.

In Chapters II and III, we extend the study to other 2'-deoxyribonucleosides, 2'-deoxycytidine and 2'-deoxyadenosine.

Chapter IV presents the results of the study of the direct effects of far UV light on thymidine, a project designed to complement the gamma study and hopefully to bring additional insight into the mechanisms of formation of those products common to both radiation energies. In particular, the mechanisms of the formation of the spore photoproduct, a lesion known to be formed in DNA in vivo, have been elucidated.

The study of the direct effects of gamma radiation on thymidine and 2'-deoxycytidine revealed the formation of several new products. Chapter V reports an analysis of the configurational and conformational properties of these molecules.
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INTRODUCTION
1.1. Deoxyribonucleic acid.

Arguably, the most interesting, and certainly the most important molecule known today is deoxyribonucleic acid (DNA). Not only is DNA at the origin of all biological processes by providing the blueprint for nature's catalysts, the enzymes, it also contains all the genetic information necessary for the faithful reproduction and continuation of species. Despite the immense complexity of the biological functions carried out by this molecule, its structure is surprisingly simple. Before discussing the effects of high energy electromagnetic radiation on DNA and its biological consequences, a quick reminder of its structure may be useful.

Complete acid hydrolysis of DNA shows it to be composed of inorganic phosphate, 2-deoxy-D-erythro pentose and four heterocyclic bases, two of which are structurally related to pyrimidine, thymine (T) and cytosine (C), and two related to purine, adenine (A) and guanine (G). With certain minor exceptions, this basic analysis holds true for all DNA, whatever its origin, from bacteria to primates. Acid hydrolysis reveals also that there exists a clear parity between certain bases, that T=A and C=G and that the ratios of pyrimidine bases to purines are close to unity. The base content of a particular DNA is generally characteristic of a particular origin and between species can vary greatly, from 25-75 mole % C+G. This variation is far more important for bacterial cells than for mammalian cells and generally in the latter group clusters between 55-65 mole % C+G. For the DNA of the bacterium E. coli commonly used in DNA research the mole fractions of the nucleobases are approximately equal.

DNA has been shown to be a heteropolymer consisting of different subunits called nucleotides. Each nucleotide may itself be further broken down into a phosphate group, a molecule of 2-deoxyribose, and one of the four bases. These subunits are linked by phosphodiester bonds making use of the C5' and C3' alcoholic functions of the furanose. We may picture DNA as consisting of a sugar-phosphate backbone to which are attached the nucleobases in the C1' position of the sugar (Figure 1).

A landmark in our understanding of DNA structure and function was the discovery by Watson and Crick (1) of its double-helical structure. X-ray diffraction analysis of DNA revealed it to consist of two complementary
Figure 1: The structure of a strand of DNA.
polynucleotide chains in part held together by hydrogen bonds between the bases, T bound to A (2 H-bonds) and C to G (3 H-bonds) (Figure 2). The genetic

![Hydrogen bonding between complementary DNA bases.](image)

Figure 2: Hydrogen bonding between complementary DNA bases.

message is contained in the order of the bases along the length of the DNA molecule. The bases are read in groups of three (codons), each coding for a particular amino acid in protein (enzyme) synthesis. The amount of genetic information carried by a DNA molecule is therefore directly proportional to its length. An average length protein of about 40 000 daltons requires a gene of approximately 1000 bases. For a cell as simple as that of _E. coli_, there are between 3000-4000 genes corresponding to a molecular weight of approximately $2.5 \times 10^9$ daltons.

I.2. The effects of high energy electromagnetic radiation on living system.

The deleterious effects of exciting (UV) and ionising (gamma and X-ray) radiations on living systems have been known for some time. Exposure of living tissue to high energy radiation results in cell inactivation, mutagenesis, cell lethality and cancer. When considering the cause of these effects at the cellular or even molecular level, it is important to draw a clear distinction between the two types of radiation.
1.2.1. The effects of UV radiation.

There has been considerable scientific interest in the effects of solar radiation on living systems for a number of years. This interest initially concerned a desire to understand chemical and biochemical mechanisms involved in the cytotoxic and mutagenic effects of solar energy, and in particular its role in the production of skin cancer and skin ageing. The discovery of various modifications of the genetic material lead naturally to the discovery of cellular repair mechanisms for these lesions and their genetic control. The knowledge obtained in these fields brought the advent of phototherapy making use of certain exogenous photosensitisers for the treatment of skin diseases such as psoriasis.

As far as the dangers presented by exposure to solar energy are concerned, we must consider the range of wavelengths which actually reach the earth's surface. The UV spectrum is generally divided into three wavelength bands, >315 nm denoted as UVA, 280-315 nm as UVB and <280 as UVC. The latter band is of no biological interest since the ozone layer surrounding the earth absorbs this region at 100%. The former two bands are however important. The cut-off frequency of ozone is 280 nm and hence the UVB range is of concern, although its intensity at the earth's surface is low at sea level. Although this range of the spectrum is the most efficient in inducing deleterious biological effects, the presence of intracellular endogenous photosensitisers means that the UVA band is also of importance, in some cases more so since it is capable of biological damage but does not induce nature's warning sign, sunburn. The effects of UVA on mammalian skin and cells have recently been reviewed (2), and those of solar radiation have recently received considerable attention from the popular scientific press (3).

For UV irradiations the action spectra, that is the survival rate of cells exposed to a particular dose of radiation with varying wavelength, coincide well with the absorption spectrum of DNA showing a maximum around 260 nm. Apart from a few aromatic amino acids, DNA is the only cellular chromophore absorbing at this wavelength, the fatty acids which constitute the cell membrane are effectively transparent in this region. A breakthrough in our understanding of the lethal and mutagenic effects of UV radiation on living cells was the identification of the pyrimidine-pyrimidine cyclobutane dimers (Pyr<>Pyr) as the major DNA lesions (4-7). Figure 3 shows the six
possible stereoisomers of the homodimer of thymine formed by the solid state or photosensitised solution UV irradiation of thymine. The high stability of these dimers under the acidic conditions used for DNA hydrolysis has allowed the detection of the cis/syn stereoisomer as the only sterically possible isomer in UV irradiated native DNA (the trans/syn isomer may be formed in slightly denatured DNA (8)).

Pyrimidine cyclobutyl dimers are not responsible for interstrand crosslinks but in fact occur uniquely between adjacent pyrimidine bases on the same strand. Hall and Larcom (9) recently demonstrated that thy$\leftrightarrow$thy dimers blocked restriction endonuclease activity at sites containing adjacent thymine residues. In the great majority of cases the pyrimidine dimers are by far the most important UV-induced DNA lesion. If unrepaired, a single cyclobutane dimer represents a lethal event in bacterial or phage genomes. This lesion has been shown to quantitatively block polymerisation and therefore replication by
E. coli DNA polymerase I (10). Pyrimidine dimers were also recently shown to inhibit semi-conservative DNA synthesis (11).

There are two other UV-induced DNA base lesions which have been shown to have important biological implications, the first of which, the so-called spore photoproduct, was first detected in the UV-irradiated spores of Bacillus megaterium by Donellan and Setlow (12) and was later identified by Varghese (13) as 5-thyminyl-5,6-dihydrothymine (Figure 4). This product has also been shown to be formed in frozen suspensions of E. coli cells (13).

The relative yields of Pyr<>Pyr and the spore photoproduct are strongly dependent upon the degree of hydration of the irradiated DNA. The formation of the former product would appear to be favored by low humidity, showing a maximum around 35% relative humidity. Recently Gould (14) showed that although their total water content may be high, bacterial endospores maintain a very low cytoplasmic water content, hence favoring spore photoproduct formation.

Recent studies on the survival of spores in space have shown that spores resist vacuum treatment at pressures of 3x10^{-7} Pa for 20 days (15). The same study obtained the UV action spectrum for B. subtilis spores in the range 180-300nm in aqueous suspension (16). In vacuo the response to irradiation was high and was found to be ten-fold that of spores in aqueous suspension. The in
vacuo conditions certainly enhance the contribution of the direct effects of the irradiation producing thymidine dimers but primarily the spore photoproduct. Not only is the importance of the spore photoproduct strongly dependent upon the external environment of the spore but also upon its germination cycle. Germination is accompanied by a distinct change in the photochemical reactivity of spore DNA, after which it appears that the predominant photoproduct changes from the spore photoproduct to the cyclobutane type dimer. This change was also shown to proceed via a highly UV-resistant stage where the yields of both the pyrimidine dimers and the spore photoproduct are very low which has been suggested to result from a change in the DNA conformation. The cyclobutane dimers are favored by high relative humidities showing a maximum between 70-100%. The sharp drop in the yield of the Pyr<>Pyr with decreasing relative humidity has been associated with the change in DNA conformation from the B to the A form at around 75% and from A to a disordered form below 65%.

The second important class of photoproduct is the group of pyrimidine (6-4) pyrimidone photoadducts, Pyr(6-4)Pyo (Figure 5). This group of products, generally named pyrimidine adducts to distinguish them from the cyclobutyl dimers referred to as the pyrimidine dimers, were first isolated by Varghese and Wang (17) from the acid hydrolysates of UV irradiated calf thymus DNA and identified by the same workers on the basis of UV, IR, NMR and mass spectral data (18). The formation and biological importance of pyrimidine adducts in
DNA have been reviewed by Patrick (19). Like the pyrimidine dimers, the pyrimidine adducts have been shown to block polymerisation by DNA polymerase I (11).

The major photoproduct formed upon exposure of DNA to 254 nm UV light is the cyclobutane pyrimidine dimer and has until recently been considered to be the most likely candidate for the UV-light-induced cytotoxic as well as premutagenic lesion in bacteria (20) due to its abundance (being formed in yields at least ten times greater than any other photoproduct in DNA at a dose of up to 100 J/m² (21)), the existence of photoreactivation specific for the cyclobutane dimers, and due to the fact that repair deficient strains unable to excise dimers are hypersensitive to exposure to UV light. In E. coli photoreactivation reverses the lethal as well as most of the mutagenic effects of UV light exposure. Recent evidence has shown, however, that the cyclobutane dimers may well be the major cytotoxic lesion, but that it is unlikely to be the only major premutagenic lesion induced by UV light. The evidence obtained leading to this conclusion also points to the involvement of the pyrimidine adducts as the primary premutagenic lesion and this evidence has been recently reviewed by Franklin and Haseltine (22). In spite of these arguments, it is clear that the nature of premutagenic lesions is very much open to debate and the subject of intense current research.

In addition to DNA base modifications, two other lesions have been shown to occur under exposure to UV light. DNA strand breaks have been shown to be produced in Chinese hamster cells exposed to fluorescent light (310nm) (23) and in human embryonic fibroblasts (24) by exposure to fluorescent lamps, sun-lamps and the sun. The third type of DNA lesion induced by exposure to UV light is covalent bonding to protein molecules. Various model studies have demonstrated photobinding between thymine and lysine (25), the binding of UV light induced photoproducts of tryptophan to DNA (26), and photochemical reactions between carboxylic acids and thymine (27).

1.2.2 The effects of ionising radiation

If the deleterious effects of UV radiation may be primarily linked to chemical modification of the genetic material, ionising radiation is far less discriminatory in its mode of attack. Cell death may be the result of the modification of a number of target molecules including membrane tissue as well as DNA. Modification of cell protein in the form of enzymes undoubtedly occurs
but such structural changes do not appear to have important consequences for
the cell. The mutagenic and carcinogenic effects of ionising radiation are
probably due to chemical modification of the genetic material, DNA. Another
distinction to be made between UV ionising radiation is the topicality of the
adverse effects. UV irradiation is not very penetrating and exposure of higher
organisms results for the most part in dermal manifestations such as erythema
(sunburn) and in serious cases skin cancer, although clearly the consequences
are more serious for microorganisms. On the other hand, exposure to ionising
radiation may result in functional disorders almost anywhere in the body
including bone cancer (28), the most sensitive areas being those where
cellular proliferation is highest. Indeed this is the basis for the
radiotherapy of cancers.

If the types of radiation induced damage occurring in the genetic material
are several, including base modifications, base free sites, strand breaks,
DNA-protein crosslinks and crosslinks formed between strands of a particular
DNA molecule, the mechanisms by which these aberrations are formed are also
diverse.

We should firstly draw a distinction between the possible modes of
degradation of a substrate exposed to ionising radiations. These effects are
generally divided into two broad groups, the direct and the indirect effects.
The primary effect of an ionising radiation on an aqueous solution containing
a target substrate is the radiolysis of the water which generally exists in a
large excess. The absorption of gamma radiation by a water molecule induces
excitation and ionisation processes.

\[
\begin{align*}
    H_2O & \rightarrow H_2O^* \\
    H_2O & \rightarrow H_2O^+ + e^-
\end{align*}
\]

These primary species then decompose to give secondary radicals.

\[
\begin{align*}
    H_2O^* & \rightarrow H^+ + OH^-
\end{align*}
\]

\[
\begin{align*}
    H_2O^+ + H_2O & \rightarrow H_3O^+ + OH^-
\end{align*}
\]
The electron ejected by the initial photon absorption is rapidly hydrated. The secondary radical species above subsequently undergo combination and/or dismutation reactions.

\[
\begin{align*}
2OH & \rightarrow H_2O_2 \\
e^-_{(aq)} + H_3O^+ & \rightarrow H^* + H_2O \\
2e^-_{(aq)} + 2H_2O & \rightarrow H_2 + 2OH^* \\
e^-_{(aq)} + H^* + H_2O & \rightarrow H_2 + OH^- 
\end{align*}
\]

The primary products resulting from this radiolysis, \(OH\), \(H\) radicals and aqueous electrons, although reduced in concentration by the latter reactions have a significant steady-state concentration and attack any solute present inducing chemical modifications. These are termed the indirect effects of the incident ionising radiation. Alternatively, the substrate may itself interact directly with the incident radiation resulting in ionisation and degradation with the interaction or otherwise of neighbouring solvent or substrate molecules resulting in a direct effect. The indirect effects may be studied by final product analysis, the \(G\) values of the major degradation products being high and the mixtures being relatively simple. The intermediate radical species formed by the addition of a water radiolysis radical product to the substrate may also be studied, either kinetically by pulse radiolysis techniques, or structurally by various spin-trapping techniques whereby a secondary substrate reacts with the primary unstable substrate radical to form a relatively stable radical which may be analysed by ESR spectroscopy.

With few recent exceptions, the studies carried out on the base lesions induced in model compounds, DNA \textit{in vitro} and in cellular studies have focussed their attentions on the indirect effects of the incident radiation. This has been for two reasons primarily, firstly, and most importantly, the cellular environment has been considered an essentially aqueous system and the indirect effects are therefore expected to predominate, and secondly and purely fortuitously the yields afforded by the indirect effects in model studies are generally far higher than those of the direct effects and hence the indirect effects pose fewer experimental problems.

The indirect effects have been well-documented from the point of view of final product analysis and various kinetic and spin-trapping experiments. The most studied lesion induced in DNA is undoubtedly the strand break. This may be a single (SSB) or a double strand break (DSB). In order for a double strand
break to occur, it is generally assumed that an ionising event must occur in each strand at close proximity. The microscopic distribution of ions generated by radiation is not uniform despite a homogeneous macroscopic appearance. Although most ionising events are distinct, being separated by about 500 nm, a fraction of the total ionising events exist in clusters of 1 to 2 nm in diameter called spurs. The idea of spurs has been proposed to explain the formation of DSB's (29). The base modifications induced by ionising radiation on nucleic acid components have been widely studied (30), some of which have been detected in DNA in vitro and in vivo (31-33).

It has been observed in several studies that a small fraction of the DNA of cells exposed to ionising radiation resists the standard methods to isolate it from other cellular components, in particular nucleoproteins (34). These observations strongly suggested the formation of DNA-protein crosslinks, and indeed Hawkins (35)(36) later managed to detect and quantify this crosslinkage in gamma irradiated bacteriophage.

The direct effects have attracted less attention, and their study has for the most part been limited to the study of the radical species formed when working with crystalline or frozen aqueous samples. For practical reasons, the study of the direct effects via analysis of the final degradation products has not been made in detail, and indeed we may cite only a handful of references.

1.2.3. DNA repair systems.

Given that we are exposed to a constant and non-negligible background of damaging radiation in the form of sunlight, cosmic rays, and radiation resulting from disintegration of natural and artificial radioisotopes especially in certain areas of high natural abundances of radioactives ores, to which we must now add such sources as radiation from reactors and particle accelerators not to mention X-rays, and accepting the overwhelming evidence of the mutagenic effects of these radiations, we may ask ourselves why are there so few mutants? The answer is that fortunately the cell has devised an intricate and highly efficient system of repair for errors introduced into the genetic code be it by radiation or chemically induced modification of the DNA molecule or natural errors made at the time of replication by DNA polymerase. Although
this enzyme itself repairs most of its initial mistakes (approximately one in $10^6$ incorporated nucleotides) via its 3'-5' exonuclease activity in an initial proof-read, there remains approximately one error for every $10^8$-$10^{11}$ nucleotides which is remarkable considering that for the bacterium E. coli having in the region of $3.8 \times 10^6$ base pairs this corresponds to about one error in every 10-1000 cell divisions.

The better known repair mechanisms are termed pre-replicative indicating that the error is repaired before DNA replication and is therefore not transferred to the cell's progeny. Several types may be distinguished. The first includes the straightforward repair of commonly occurring faults such as strand breaks, base loss resulting in apurinic or apyrimidinic sites, and incorporation of structurally similar substrates such as uracil for thymine or 06-methylguanine.

DNA strand breaks are not only the result of the effects of irradiation and several workers studying the repair of these lesions have detected back-grounds of 300-700 strand breaks per cell. Considering the importance of this lesion it is essential that the cell have an efficient system for its repair, indeed virtually all mammalian cells have the ability to rejoin radiation-induced strand breaks. If the strand break represents a 3'-OH and a 5'-phosphate, the lesion is repaired directly by a DNA ligase re-establishing the 3'-5' phosphodiester bond. Base loss is a phenomenon not unique to radiation exposure, indeed DNA spontaneously loses approximately 10000 bases from its genome per day which are replaced by a specific insertase depending on the particular purine or pyrimidine triphosphate substrate to be used. Erroneously incorporated uracil instead of thymine is excised by the enzyme uracil-DNA-glycosylase. It should be noted that uracil may occur in DNA by chemically or radiation induced deamination of cytosine as well as by replicational error. 06-methylguanine is repaired by transfer of the methyl group to a cysteine unit of the enzyme trans-methylase.

A second type of pre-replicative DNA repair system is more complicated and is comprised of a series of steps each associated with a particular enzyme activity. The best understood system is the 'cut and patch' method and is associated with the repair of mismatched or modified bases. Firstly the altered base is removed by a glycosylase specific for a particular lesion resulting in an AP site. The next step is an incision at this site by the enzyme AP endonuclease which creates 3'-OH 5'P extremities. An exonuclease
subsequently digests a stretch of the broken strand adjacent to the nick (or perhaps via the exonuclease activity of DNA polymerase reported by Deutscher and Kornberg (37) as suggested by Trgovcevic and Kucan (38)), DNA polymerase reconstitutes the empty region produced using the intact strand as a template, the final phosphodiester stitch being made by ligase.

It has been shown that at least two mechanisms exist for the repair of cyclobutane dimers in bacterial cells. One involves the excision repair described above (39) with a specific UV-endonuclease making the initial incision 5' to the photoproduct region (40) and digestion of a ten-nucleotide stretch of the modified strand before DNA polymerase action. *E. coli* also has a DNA photolyase which reverses cyclobutane dimers directly with absorption of visible light. This system is referred to as photoreactivation and is specific for the cyclobutane dimers having been shown to be inactive against pyrimidine-(6-4)pyrimidone adducts (41). The pyrimidine adducts are however repaired by an excision repair which in *E. coli* is controlled by *uvr*A, *uvr*B and *uvr*C genes (42). It has been shown recently that the pyrimidine adducts are removed from mammalian cells more efficiently than the cyclobutyl dimers (43). The spore photoproducts have also been shown to be removed by two distinct mechanisms involving two specific genes in *Bacillus subtilis* spores. As for the cyclobutane dimers, there exists an excision repair mechanism that has been shown to be controlled by the same genes which regulate cyclobutane dimer excision in vegetative cells. There is also strong evidence for the direct monomerisation of the spore photoproduct to two thymines by a light-independent "spore repair" process in *Bacillus subtilis* (44).

Repair of DNA-protein crosslinks is known to occur although it would appear that UV induced crosslinks are not repaired in the same way as those formed by gamma irradiation. Chiu et al recently (45) showed that differential processing of ultraviolet and ionising radiation induced crosslinks in Chinese hamster cells occurs. They showed that although both types of crosslink could be detected in these cells, only those induced by ionising radiation were released during post-irradiation incubation, and that release of UV induced crosslinks could not even be induced by prior irradiation with gamma rays. It was concluded that the nature of the crosslink or other factors must be substantially different for the repair system to recognise one type but not the other.
From the above discussion, it is clear that the study of the deleterious effects of high energy radiation on living systems has attracted a great deal of research in recent years. It is also widely accepted that in order to understand the processes involved in the induction of adverse pathological manifestations, a detailed knowledge of those occurring on the molecular scale is essential.

The carcinogenic and mutagenic effects of these radiations are linked to alterations of the genetic material. Owing to the complexity of the substrate involved, DNA, the study of nucleic acid model compounds represents a necessary starting point towards an understanding of the processes occurring at the cellular level. For the indirect effects of the incident radiation, studies of the radical species formed and those of the resulting diamagnetic products have been shown to be highly complementary approaches. For the direct effects, although the primary radicals formed have been widely studied using the ESR and ENDOR techniques, there is almost a complete lack of information concerning the nature of the final products.

The motivation for the present study was a desire to rectify this paucity, at once providing complementary information to primary radical studies, and also revealing the nature of nucleic acid modifications as a precursor to the search for these lesions at the cellular level. For this purpose, the irradiation of 2'-deoxyribonucleosides in frozen aqueous solution appeared to us an ideal model system, being compounds which lend themselves to relatively simple structural analysis and most of whose degradation products are known compounds.
CHAPTER I

THE DIRECT EFFECTS OF GAMMA RADIATION ON THYMIDINE

1.1. Frozen aqueous solution; a valid model system?

Before reporting the results of the study of the direct effects of gamma radiation on DNA and its model systems, the choice of the frozen aqueous medium as an appropriate system requires justification. Let us consider the features we require of an ideal model system, and judge to what extent frozen aqueous solutions fulfill these requirements.

Firstly, it is clearly essential that we isolate the direct effects from the indirect effects. In view of the negligible degradation, for a given radiation dose, by the direct effects compared to the indirect effects in liquid aqueous solutions, such a separation is not necessary in studies of the indirect effects. In studies of the direct effects, however, the efficiency of the mobile water radiolysis products in mediating radiation damage to nucleic acid substrates requires that our model system be highly effective in eliminating the indirect effects.

We might suspect that the best system for eliminating the indirect effects completely is the anhydrous solid phase, and this is undoubtedly so, however we would unfortunately lose certain important advantages of frozen aqueous samples (vide infra). Irradiation of a frozen aqueous solution obviously leads to the formation of water radiolysis products, we must therefore satisfy ourselves that these highly reactive species are unable to diffuse to the substrate. During freezing, an aqueous sample undergoes phase separation as the growing ice crystallites exclude the substrate, forming aggregates of very high local concentration in the interstices of the crystal (46). These aggregates have a well-ordered microcrystalline structure with planar aromatic molecules, like DNA bases, undergoing vertical stacking and horizontal hydrogen bonding contributing to the microcrystalline structure of these aggregates. The configuration of these aggregates provides good overlap of the \( \pi \)-orbitals and permits intermolecular charge transfer phenomena to occur.
Although several studies have provided evidence in alkaline or acid glasses that the indirect effects largely outweigh the direct effects, Gregoli et al. (47) demonstrated that in frozen neutral solutions above 135 K (the temperature at which \( \text{OH}^+ \) radicals in the ice crystallites decay) the yield of thymidine 5'-monophosphate (dTMP) radicals shows a linear dependence on the solute concentration strongly suggesting direct action only. The authors also pointed out however that the linear dependence on concentration could also be explained by equal rates of formation and destruction of dTMP radicals by hydration water resulting in a local indirect effect, although they estimated this contribution to be minimal for the reason that the water radicals formed are likely to be present as clusters and are expected to mutually deactivate before diffusion to the substrate may occur. Nevertheless, the contribution of a local indirect effect will depend strongly on the state and mobility of the hydration water molecules bound to the substrate. The important results of Mathur-De Vrè and Bertinchamps (48)(49) indicate that non-negligible quantities of NMR visible water protons exist in frozen aqueous solutions of nucleic acids. This hydration layer consists of some 10 water molecules per nucleotide (50) which remain just as mobile at 196 K as at 273 K. As if to underline the controversy that reigns over this problem, Woldhuis et al. (51) ruled out the participation of \( \text{OH}^+ \) and \( \text{H}^+ \) radicals and aqueous electrons in the inactivation of \( \Phi X174 \) DNA by gamma rays in frozen aqueous solution at 253 K deducing that inactivation occurs mainly by reaction of dry electrons.

In fact the only true indication of the existence of any contribution from the indirect effects in the present study and their relative importance lies in the identification of degradation products directly attributable to the interaction of water radiolysis species with the substrate. As will be shown later, such products are quantitatively much less important than those readily accounted for by the direct effects and indeed may themselves also result from the latter.

We also require of our model system that the substrate experience conditions resembling those experienced by DNA in vivo. It is considered that the hydration degree of cellular DNA is far from that of an ideal aqueous solution and in this respect the frozen aqueous system may imitate these conditions fairly well. Furthermore, in native DNA, the nucleosidic components exist in an ordered arrangement, the helical
tertiary structure being stabilised by interstrand hydrogen bonds between the complementary nucleobases, and perhaps more importantly by a number of cooperative weaker bonds such as intrastrand stacking forces (Van der Waals forces and London dispersion energies) arising from interaction of the heterocyclic bases as they stack vertically in an ordered manner, and electrostatic forces between the solvent and the externally orientated sugar-phosphate backbone. This stacking in DNA results in charge transfer processes under the effects of ionising radiation. The aggregates formed upon freezing aqueous solutions show similar phenomena.

The use of the frozen aqueous solution system may however present one disadvantage. Although we have no direct evidence to suggest that it be the case, the fact that the sample is irradiated at temperatures much lower than those encountered in vivo may affect the course of the chemical reactions undergone by the initially generated radicals and these may differ from those that may take place under physiological conditions.

The above discussion may be summarised as follows:

- The primary ionising events in DNA are the same in vivo and in the frozen or dry states, unlike the indirect effects mediated in dilute aqueous solution where the presence in vivo of a variety of secondary solutes may also be involved in the formation of radiation-induced modifications of DNA.

- Unlike the dry state, primary DNA radical ions can enter conversion reactions with hydration water molecules both in vivo and in the frozen aqueous state.

- The radiolytic processes induced in the frozen state are generally accepted to result from a direct effect of the incident radiation.

In conclusion, the frozen aqueous system would appear to represent the best model system for the study of the direct effects of ionising radiation on nucleic acids.
1.2. PRIMARY RADICAL PROCESSES FOR THYMINE AND ITS DERIVATIVES

In this section, we shall review briefly current knowledge of the nature and behaviour of certain major primary thymine radicals in DNA and the isolated base and base derivatives. We shall limit our discussion to events occurring under neutral conditions.

1.2.1. The thymine $\pi$-anion

Capture of an electron by the thymine residue in DNA and thymine derivatives yields the radical $\pi$-anion, having a resonant structure with highest negative charge density residing at 04 and highest unpaired spin density being sited at C6. Apart from intermolecular electron transfer, the primary process leading to restitution of the initial charge state is protonation, generally at C6 although addition at heteroatoms may also occur (Figure 1.1).

![Figure 1.1](image1.png) Protonation pathways for the thymidine radical $\pi$-anion.

In the presence of oxygen, both the pristine $\pi$-anion (52)(53) and its C6 proton adduct may be scavenged (Figure 1.2).
1.2.2. The thymine radical cation

The two major processes, apart from electron return, leading to regeneration of the initial charge state of the radical are deprotonation and anion addition. For thymine, the preferred site of deprotonation is at N1 with a minor contribution from proton loss at the methyl group. In N1-substituted thymines, N1 deprotonation becomes impossible and deprotonation at the methyl group becomes the predominant process (Figure 1.3).

Figure 1.2: Scavenging by molecular oxygen of the thymidine radical $\pi$-anion and its protonated derivative.

Figure 1.3: Deprotonation pathways of thymidine radical $\pi$-cations.
The thyminyl radical (I) may also be formed via superexcitation whether N1 is substituted or not.

In the absence of co-solutes, the main anion addition process involves OH$^-\text{ transfer from water (Figure 1.4). The addition of hydroxide anion to thymine } \pi\text{-cations has been demonstrated by Sevilla and Engelhardt (54), and to the 5'\text{-TMP cation by Gregoli et al (55) in frozen aqueous solution.}

![Figure 1.4: Hydroxyl anion addition to the thymidine radical $\pi$-cation.](image)

1.2.3. The final degradation products resulting from the direct effects of gamma radiation on thymidine

Table I.1 lists the degradation products of thymidine isolated during this study, giving references where these are known compounds.

1.3. CHROMATOGRAPHIC SEPARATION OF THYMIDINE DEGRADATION PRODUCTS

The search for and characterisation of DNA lesions created by exposure to ultraviolet and ionising radiations has always relied heavily on chromatographic techniques. In the early days, the most frequently used systems were paper and thin layer chromatographies for analytical work, and thick layer silicagel plates (2mm) for preparative separations. The former techniques still hold an important place in routine analysis. The development of high performance liquid chromatography (HPLC) represented a landmark in many fields, and notably in the domain of nucleic acids research. The technique was readily adapted to the problems posed by these studies, namely those of small quantities requiring high sensitivity, and the highly complex mixtures habitually encountered, often involving diastereomers, necessitating high
<table>
<thead>
<tr>
<th><strong>TABLE I.1</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PRODUCTS RESULTING FROM THE GAMMA RADIOLYSIS OF THYMIDINE IN FROZEN AQUEOUS SOLUTION</strong></td>
</tr>
</tbody>
</table>

1. Thymine. 
2. 5,6-dihydrothymine. 
3. 5,6-dihydrothymidine (5R, 5S) (60). 
5. 5-hydroxy-5,6-dihydrothymidine (5R, 5S) (57). 
7. 5-hydroxymethyl-2'-deoxyuridine (59). 
9. 1-(\(\alpha-L\)-threo-furanosyl)thymine. 
10. Thymidine 5'-aldehyde. 
11. 2'-deoxyribonolactone. 
12. 5',6-cyclo-5,6-dihydrothymidine (six diastereomers). 
15. dThd(\(\alpha-\alpha\))dThd. 
16. dThd(3-5)hdThd (5R, 5S). 
17. dThd(5'-6)hdThd (two diastereomers). 
18. dThd(\(\alpha-3\))dThd.
resolution. Except in certain isolated instances, the use of thick layer plates is practically obsolete avoiding the long and often inefficient extraction process.

The absence in the early 1970's of HPLC techniques did not significantly retard research on the indirect effects of UV and ionising radiations on nucleic acids owing to the high yields of degradation products obtained. However, the direct effects presented problems practically insurmountable using the early chromatographic methods. A complete resolution of the degradation product mixture obtained from the gamma irradiation of thymidine in frozen aqueous solution necessitates the use of in some instances four different HPLC columns and twice as many solvent systems.

In this section we shall outline the general analytical approach used in the study of the direct effects on thymidine. Although elution profiles are likely to differ from one column to another, even when the same phase is used due to variations in column packing conditions, careful solvent preparation and the use of a particular column generally yield highly reproducible separations.

1.3.1. The general analytical approach

Fifteen days continuous irradiation with an overall dose of 4 MGy of gamma radiation results in approximately 10% thymidine degradation, the bulk of this being comprised of five principal products, namely 5,6-dihydrothymidine, thymine, 5,6-dihydrothymine, 5-hydroxymethyl-2'-deoxyuridine and the thymidine cyclobutane dimers.

The small remaining fraction represents twenty or so quantitatively minor products. Before an efficient resolution of the mixture may be effected, the remaining bulk thymidine must be removed, if not totally, at least to an extent where its concentration is comparable to that of the most important degradation product. This is done very simply by crystallisation from hot ethanol. After two or three concentrations, 80% of the undegraded thymidine may generally be removed. The mixture is then fractionated very crudely on a preparative reverse phase HPLC
column, and each fraction subsequently submitted to semi-preparative purification.

The complexity of the mixtures obtained does not allow for the complete resolution by any one column and solvent system. It is therefore necessary to fractionate a second time on one column type and then reinject onto a different stationary phase in the hope that no two products present identical retention characteristics on both columns. Because of the high resolution generally obtained, and the increased solvent versatility associated with reverse phase systems, the first semi-preparative fractionation is made using an octadecasilyl silicagel column associated with a water/methanol system. The fractions obtained are then evaporated to dryness and reinjected onto a silicagel column in the partition mode.

Generally, these two columns between them are capable of resolving the mixture entirely, however it was necessary in one instance to resort to the use of a third phase type - an N-capped Waters Associates ODS3 column - for the separation of thymine and one isomer of 5',6-cyclo-5,6-dihydrothymidine, taking advantage of the removal of the underivatised silyl groups which may act as a weak cation exchange support.

We shall now describe in more detail the individual stages in the resolution of the degradation product mixture.

1.3.2. Analytical thin layer chromatography

Two dimensional thin layer chromatography (t.l.c.) is a rapid and efficient analytical tool. At each stage of an organic synthesis or a purification procedure, a t.l.c. analysis was carried out to monitor the composition of the reaction mixture or purification fraction. For nucleoside and free base products, two standard solvent systems were used:

Solvent I : Chloroform-methanol-water (4:2:1) (100 ml of lower phase plus 5 ml of methanol)

Solvent II : Ethyl acetate-propan-2-ol-water (75:16:9)
Figure 1.5 shows a thin layer plate of the thymidine degradation mixture for which the majority of the undegraded thymidine has been removed by precipitation. Because of superposition of many spots, we have marked just the quantitatively most important.

1.3.3. High performance liquid chromatography (HPLC)

1.3.3.1 Reversed phase HPLC

As mentioned above, the initial preparative and the first analytical fractionations are carried out using a reversed phase system. For the preparative separation, the irradiation mixture (after precipitation of the bulk undegraded thymidine) is injected onto a preparative apparatus using a 90/10 water-methanol solvent. The eluent is fractionated and evaporated to dryness. The column is washed thoroughly with methanol in order to remove highly retained products. The fractions eluted before and after the thymidine peak are reinjected separately onto a semipreparative reversed phase column using the appropriate solvent systems. The early eluting fraction is separated using a polar solvent, water, and the retained fraction using a methanol containing solvent, usually 95/5 or 90/10 to speed up the separation. We obviously lose in resolution but the separation would appear sufficient given that the fractions are reinjected onto a silicagel column subsequently.

It is worth making some general remarks on the retention times of modified thymidines. Firstly, with the exception of the cyclobutane-type thymidine dimers, all dimeric products are better retained on the reversed phase column than thymidine and the monomeric derivatives, and may be explained by the fact that all other dimers contain at least one unsaturated pyrimidine ring conferring higher hydrophobicity than saturated derivatives. This facilitates the separation of these products which are formed in far lower yields and as a result are not masked by the monomeric compounds. In addition, we are able to concentrate these minor products and are therefore less likely to miss those which are interesting from a mechanistic standpoint but which are only formed in trace amounts.
Figure 1.5: Two dimensional silicagel thin layer chromatogram of the thymidine degradation mixture upon gamma irradiation in frozen aqueous solution.

1. (-) cis-anti dThd <> dThd
2. (+) cis-anti dThd <> dThd
3. cis-syn dThd <> dThd
4. spore photoprodut
5. trans-anti dThd <> dThd
6. 5-hydroxymethyl-2'-deoxyuridine
7. S5 and S5 5,6-dihydrothymidine
8. thymidine
9. thymine
Secondly, for monomeric products, the saturation of the 5,6-double bond reduces the retention time of the product with respect to thymidine, this time due to a reduction in the hydrophobicity upon saturation.

1.4. STRUCTURAL ANALYSIS OF THE PRODUCTS FORMED BY THE DIRECT EFFECTS OF GAMMA RADIATION ON THYMIDINE

Apart from the 5',6-cyclo-5,6-dihydrothymidines, all monomeric products of the direct effects of gamma radiation on thymidine are known compounds and their identification was made by comparison of their spectroscopic characteristics with literature values. The same holds for the cyclobutane dimers. The spore photoproduct has been characterised as the free base analogue (62) but the spectral analysis of the dimeric nucleoside has not yet been made. The $^1$H NMR spectrum of one of the isomers has been recorded at 600 MHz (63). During our study, both isomers have been obtained in sufficient quantities to allow extensive NMR analysis, the proton assignments having been made by homonuclear two dimensional correlation spectroscopy and using these assignments, the $^{13}$C signals could be made by heteronuclear $^{13}$C-$^1$H chemical shift correlation spectroscopy. The results of this analysis will be given in this section since these data have not been published elsewhere.

1.4.1. Modern pulse methods in high resolution nuclear magnetic resonance spectroscopy

The replacement of continuous wave NMR techniques by Fourier transform pulse techniques not only revolutionised the NMR technique in terms of resolution and sensitivity, it also brought the advent of numerous multi-pulse methods which extend the versatility and power of the nuclear magnetic resonance technique to such a point that it is today perhaps the most powerful analytical tool available. Before discussing the application of NMR to the structural analysis of the new compounds isolated from the gamma irradiation of various nucleoside derivatives mentioned in this chapter and in Chapter IV, it is worthwhile giving a brief introduction to two-dimensional NMR techniques.
1.4.1.1. Two-dimensional NMR

A common feature of most modern pulse methods in high resolution NMR experiments is the time sequence preparation-evolution-detection (Figure 1.6). The preparation period is a delay time which is long compared to the longitudinal relaxation time of the nuclear spins in order that the spins attain their equilibrium orientations according to the direction of the applied magnetic field \( H_0 \). At the end of the preparation period, usually a non-equilibrium spin system is created by means of one or a series of radiofrequency pulses. The pulse sequence used depends on the type of experiment.

![Figure 1.6: General time-sequence for modern NMR pulse methods.](image)

If we consider the magnetisation vector of the whole spin system \( M \) (macroscopic magnetisation) to be aligned with the z-axis under the influence of the applied field \( H_0 \), a 90° or \( \pi/2 \) pulse deflects \( M \) into the xy plane along the positive y-axis. The longitudinal or z magnetisation is transformed into transverse magnetisation. Each spin in the system has its own Larmor frequency related to the external field strength by

\[
\nu = \left( \frac{\gamma}{2\pi} \right) H_0
\]

where \( \gamma \) is the gyromagnetic ratio of that particular spin and is a function of its local magnetic environment. Each spin will precess during the evolution period at its Larmor frequency and hence \( M \) now splits into its individual components. The vectors rotating in the xy plane induce a
voltage in the receiver coil detected as the NMR signal. In one-dimen­sional experiments, the receiver signal is solely a function of the detection time $t_2$. The same time sequence of preparation-evolution­detection also forms the basis of 2D-NMR experiments with the important difference that the evolution period $t_1$ now becomes a variable. If over n experiments, we alter the value of $t_1$ by an increment $t_1$ keeping all other parameters constant, we introduce a second time variable. Two time variables imply that the data may be Fourier transformed twice, i.e. with respect to $t_1$ and $t_2$ with the result that we obtain two frequency variables.

The principle of 2D-NMR spectroscopy relies on the ability of the spin system to remember during the detection period what happened to it during the evolution period. This history can either affect the phase or amplitude of the magnetisation of those spins, which is detected during the $t_2$ period. In the case of phase modulation, the phase of the magnetisation component at the beginning of the detection period is a linear function of $t_1$. For amplitude modulation, the amplitude of the magnetisation in the xy plane is an oscillating function of $t_1$ while its phase is independent of $t_1$.

1.4.1.2. Chemical shift correlation spectroscopy (COSY)

After the preparation period, a $90^\circ$ pulse produces transverse magnetisation. The magnetisation vectors then precess in the xy plane according to their Larmor frequencies and their spin-spin coupling constants $J$. The second pulse leads, for a non-coupled spin $A$, to $t_1$-dependent modulation of the transverse magnetisation which is dependent only on $\nu A$, the Larmor frequency of $A$ and in the 2D spectrum gives a signal at $\delta A \delta A$. For the case of a non-zero coupling to a spin $X$, the $A$ magnetisation also depends on the Larmor frequency of $X$ through scalar $AX$ interaction and the 2D spectrum contains additional signals at off-diagonal coordinates centred at $\delta A \delta X$ and $\delta X \delta A$, in fact four signals are observed at $(\delta A-J)$ $(\delta X-J)$ and $(\delta X-J)$ $(\delta A-J)$.

A series of decoupling experiments would yield the same information as a 2D COSY experiment, however interpretation of the former in cases of complex overlapping of signals is often difficult. In addition, a single COSY experiment provides all the necessary information and is often less time consuming.
1.4.1.3. J-resolved 2D $^1$H NMR

For this type of 2D NMR experiment, the spin echo pulse sequence is used. Briefly, this consists of a 180° pulse half way through the evolution period such that the field inhomogeneity effects are refocussed at the beginning of the detection period. Figures 1.7 and 1.8 show the pulse sequence and vector representations respectively for the J-resolved 2D experiment using the example of an A nucleus of a homonuclear AX system. At time $t_1/2$, the doublet components have progressed by different phase angles and at the same time are broadened by field

Figure 1.7 : Pulse sequence for 2D homonuclear J-resolved NMR spectroscopy.

Figure 1.8 : Vector representation for the 2D homonuclear J-resolved NMR experiment.
inhomogeneity effects. It should be mentioned that spin vector precessions are considered with respect to an imaginary frame rotating at or near to the Larmor frequencies of the spins under consideration. The 180° pulse inverts the A1 and A2 vectors around the x-axis, but also changes the spin states of the X nucleus. The Larmor frequencies are therefore interchanged and only field inhomogeneity effects are refocussed at the end of the second $t_{1/2}$ period. This inversion of the Larmor frequencies of the spin vectors, or put another way, their velocities in the rotating frame becomes clear if we consider the spin transitions involved.

Figure 1.9: Spin transitions for spin A in an AX spin system.

Figure 1.9 shows the corresponding transitions for spin A. The X nuclei are also inverted and hence transition 1 becomes $\beta\beta \rightarrow \alpha\alpha$ corresponding to transition 2, and similarly transition 2 becomes transition 1. It is therefore clear that after the 180° pulse, the magnetisation corresponding to transition 1 will change its rate of rotation in the xy plane such that it now turns at the rate of transition 2, and vice versa.

At the beginning of acquisition the magnetisations of the two lines of spin A will not be refocussed but will be located symmetrically about the OY axis, whatever the chemical shift of A. Each will have its amplitude modulated according to $\cos 2\pi J t_1$ and phase modulated by $-2\pi J t_1$ independent of the chemical shift of A. After double Fourier transformation, the F1 dimension contains the coupling information, but since
the spins are coupled during the detection period, the second frequency variable \( F_2 \) is a function of both the chemical shift and the spin-spin coupling constant \( J \). An immediate separation of the coupling constants and chemical shifts is therefore not possible. In fact, the individual multiplets lie on a 45° inclined angle of the two dimensional density matrix. Projection onto the \( F_1 \) axis yields the multiplicity and the coupling constants while projection onto the \( F_2 \) axis yields the chemical shift data. A useful aspect of this experiment is the high resolution obtained since the spin echo refocusses field inhomogeneity effects and therefore the lines have their natural widths given by: 

\[
\Delta = 1/T_2
\]

1.4.1.4. Two-dimensional heteronuclear correlation spectroscopy

It is easily shown, on the basis of vectorial representations and consideration of various energy populations, that a pulse sequence \( 90(H) - t_1 - 90(H), 90(C) \) results in a polarisation transfer from \( H \) to \( C \) modulated by the coupling constant \( J(C-H) \). The energy level diagram for a \( CH \) system (Figure 1.10(a)) shows the relative populations at equilibrium and reflects the fourfold increased sensitivity of the proton over

![Figure 1.10](attachment:energy_level_diagram.png)

**Figure 1.10** : Energy level diagram and relative populations for a \( CH \) spin system before (a) and after (b) selective population inversion.
the $^{13}$C nucleus ($\gamma_{H}=4\gamma_{C}$). If a population inversion is created across one of the two proton transitions without significantly affecting the other proton transition then the new populations represented in Figure 1.10(b) apply. Clearly one $^{13}$C line shows a five-fold increase in

$$\begin{align*}
\begin{array}{c}
90^\circ \\
\hline \\
\hline \\
\hline \\
180^\circ \\
\hline \\
\hline \\
\hline \\
90^\circ \\
\hline \\
\hline \\
\hline \\
\frac{1}{2}t_1 \\
\hline \\
\hline \\
\hline \\
\frac{1}{2}t_1 \\
\hline \\
\hline \\
\hline \\
\Delta_1 \\
\hline \\
\hline \\
\hline \\
\Delta_2 \\
\hline \\
\hline \\
\hline \\
\text{acquisition}(t_2)
\end{array}
\end{align*}$$

Figure 1.11: Pulse sequence for $^1$H-$^{13}$C 2D heteronuclear chemical shift correlation spectroscopy.

intensity and the other a three-fold increase which is also inverted. The two-dimensional experiment uses a pulse sequence (Figure 1.11) which has the effect of selective population inversion, but achieves it for all resonances in the proton spectrum, irrespective of chemical shift. The degree of population inversion is modulated by $t_1$ the evolution period as shown in Figure 1.12, where it is evident that when the phase

$$\begin{align*}
\begin{array}{c}
\text{(a)} \\
\text{(b)} \\
\text{(c)} \\
\text{(d)} \\
\text{(e)}
\end{array}
\end{align*}$$

Figure 1.12: Representation of the effect of two 90° pulses separated by an interval $t_1$. (a) If $\Phi = 2n\pi$, the result is population inversion. (b) If the spins precess through $\pi/4$ radians, the second pulse has no effect and the $z$ magnetisation is saturated. (d) If $\Phi = (2n + 1)\pi$, the spins are returned to Boltzmann equilibrium by the second 90° pulse. The general result is that population inversion is modulated by $t_1$. 

angle is \(2\pi\) the population transfer is maximum, the two 90° pulses acting as a single 180° pulse. When \(\phi = (2n+1)\pi\) the two 90° pulses cancel each other out to yield the Boltzmann equilibrium populations. At intermediate phase angles, the degree of population inversion is incomplete leading to a cosine modulation of the population inversion as a function of \(t_1\). During the detection period \(t_2\), the nuclear magnetisations precess at the \(^{13}\text{C}\) Larmor frequencies. In the simplest case of a \(^{13}\text{CH}\) group, there are signals at \(\delta^{13}\text{C}-(1/2)J\ Hz\). After two-dimensional Fourier transformation, the spectrum contains four lines, since both proton transitions pump spin populations into or out of both \(^{13}\text{C}\) transitions. These lines have ordinates \(\delta^{13}\text{C}+(1/2)J\ Hz\) and abscissae \(\delta^{13}\text{H}+(1/2)J\ Hz\).

For chemical shift correlation, this multiplet structure is an unnecessary complication and therefore during both time intervals \(t_1\) and \(t_2\) the \(^1\text{H}\) and \(^{13}\text{C}\) nuclei are decoupled. During the evolution period, this is achieved by a 180° pulse applied to the \(^{13}\text{C}\) nuclei at the midpoint of \(t_1\) causing the diverging magnetisation vectors to refocus at the end of the evolution period. During the detection period, noise decoupling of the protons suffices. Without decoupling, the above lines have intensities which alternate in sign since a population pumped into one carbon transition always corresponds to an equal population pumped out of the other. To prevent mutual cancellation of these anti-phase signals when the doublets collapse due to decoupling, two fixed delays, \(\Delta_1\) and \(\Delta_2\), are introduced to allow 180° relative precession of the pairs of vectors before they are combined. Since \(\Delta=1/(2J)\) necessarily, we effectively minimise signals that originate from magnetisation transferred through long-range coupling, since the corresponding vectors have insufficient time to precess from the anti-phase condition and so cancel.

1.4.1.5. The NOE difference experiment

The nuclear Overhauser effect (NOE) is a change in the integrated NMR absorption intensity of a nuclear spin when the NMR absorption of another spin, with which it is at least spatially coupled, is saturated. The theory of the nuclear Overhauser effect will not be exposed here, suffice it to say that it relies upon the increased probability of a particular quantum jump of a spin system when one spin transition is
saturated. For a more complete expose of the NOE and its applications in structure analysis see (64). Experimentally, a normal spectrum is recorded involving one or a number of acquisitions. The experiment is repeated this time with saturation of the signal of interest with the same number of scans. The two spectra are then written to different files. The procedure is repeated n times and the spectra obtained added to the appropriate file. Each file is then Fourier transformed, and one subtracted from the other.

1.4.2. Structural analysis of new thymidine degradation products

Before discussing the structural assignments of individual products, it may be useful to recall some of the general trends in the $^1$H NMR spectra of 2'-deoxyribonucleosides. We shall focus our attention on the furanose moiety signals since these are generally the most important in conformational studies, and the non-exchangeable base protons are few and generally easy to distinguish. We may first make a general observation concerning the order of the chemical shift values encountered for the vast majority of 2'-deoxyribonucleosides (assuming the absence of any electronegative substituents at the hydroxyl groups which may induce considerable downfield shifts of the H3' and H5'H5'' signals), which is as follows: 2'2', >5'5', > 4 '>3'>1'.

The difficulties arise in assigning the pairs of methylene protons on C2' and C5'. Based on the arguments of Remin and Shugar (65), the downfield part of the AB pair in the ABX system is assigned as the 5' proton and that at higher field as the 5'' signal. Fraser-Reid and Radatus (66) resolved the problem for the H2' and H2'' assignments through specific deuteration studies using 2'-deoxycytidine. They observed that the value of J(2''3'), is generally approximately equal to J(3'4') whatever the sugar conformation, the pairs both constituting an equatorial-equatorial pair in the case of the 2'-endo conformation and an axial-axial pair for 3'-endo. As pointed out by Wood et al (67), this might be expected since the configurational relationship between the 2'' and 3' pair and the 3' and 4' pair is identical, one hydrogen being endo, the other exo in each pair. In addition the 2'' and 4' protons have equivalent orientations with respect to the 3'-hydroxyl group (gauche in each case) for both the 2'-endo and 3'-endo conformations, and therefore
Electronegativity effects are equivalent in each case. The $\text{H}_2'$ and $\text{H}_3'$ generally constitute a cis pair with correspondingly a $J(2'3')$ value larger than the $J(2''3')$ value. Therefore, for a primarily $2'$-endo conformation, determination of the values of the above coupling constants immediately distinguishes the $\text{H}_2'$ and $\text{H}_2''$ signals. Typical values for the preferentially $S$ conformation for $J(2''3')$ and $J(2'3')$ are 4 Hz and 6-6.5 Hz respectively. A shift towards the $N$ conformation generally results in an increase in the first with a concommittant decrease in the value of $J(2'3')$.

The anomeric proton generally presents a pseudo-triplet or a double doublet. The $\text{H}_1'\text{H}_2''$ coupling constant usually lies between 5.7-6.6 Hz. The $\text{H}_1'\text{H}_2'$ coupling is generally stronger varying from around 6.3 Hz to as high as 9.5 Hz. The $^1\text{H}$ NMR characteristics of the new dimeric thymidine adducts are listed in Table I.2.

1.4.2.1. 5',6-Cyclo-5,6-dihydrothymidine

The EI mass spectrum confirms the mass of 242 amu. The major fragmentations proceed via an initial rupture of the C5'-C6 bond which with rupture of the N-glycosidic link gives the base peak ion at 127 amu. The UV absorption spectrum of this compound also provides an important structural clue, namely that the molecule has lost the absorbance in the region of 260 nm characteristic of 5,6-unsaturation.

The configurational and conformational analysis of these products using the NMR technique is described in Chapter V. Here we shall be content to describe the general spectral characteristics of all six isomers in order to support the proposed structure. Figure 1.13 shows the 400 MHz $^1\text{H}$ NMR spectrum of Isomer I. Firstly, no vinyl proton signal is observed at its expected downfield chemical shift in the region of 7.5 ppm supporting the UV data which indicated saturation of the 5,6-double bond of thymidine. We note the appearance of a multiplet in the region of 4 ppm which we assign as the $\text{H}_6$ proton resonance. In addition, we note an upfield shift of the methyl peak compared to that of thymidine which now presents itself in the form of a doublet. The final indication of the saturation of the base is found in the observation of a one proton multiplet having a chemical shift which correlates well with that of the C5 methyne proton of 5,6-dihydrothymidine (61).
**TABLE 1.2**

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<th>dThd(α-6)hdThd I</th>
<th>dThd(α-6)hdThd II</th>
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<td>6.32</td>
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**Chemical shifts are given in ppm against TPS internal standard.**

**Coupling constants are in Hz.**
### TABLE 1.2 continued.

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<thead>
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<td>A</td>
<td>B</td>
</tr>
<tr>
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<td>6.27</td>
<td>6.32</td>
<td>6.27</td>
</tr>
<tr>
<td>δ2'</td>
<td>2.28</td>
<td>2.41</td>
<td>2.40</td>
<td>2.34</td>
</tr>
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- the 2'2'' system is degenerate and the geminal coupling constant stated is an approximate value.
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a - system degenerate.
Figure 1.13: 400 MHz $^1$H NMR spectrum in D$_2$O of Isomer I of 5',6-cyclo-5,6-dihydrothymidine.
A feature common to all isomers of 5',6-cyclo-5,6-dihydrothymidine is the observation of an effectively zero coupling between protons H1' and H2', and H3' and H4'. This is due to the induction of a severe distortion of the deoxyribose residue upon cyclisation. This distortion results in the four ring carbons C1', C2', C3' and C4' being coplanar with the effect that all trans protons now have dihedral angles of close to 90°. The same has also been observed for other known cyclonucleosides of this type, namely the 5'R (68) and 5'S (vide infra) isomers of 5',8-cyclo-2'-deoxyadenosine and 5',6-cyclo-2'-deoxyuridines (69). It is also noteworthy that we observe a long distance coupling of approximately 1 Hz between protons H2" and H4' again due to the induced distortion resulting in coplanarity of this part of the nucleoside.

Proton resonance signals were assigned by selective decoupling experiments and the chemical shifts and coupling constants of the six diastereoisomers are given in Table I.3.

1.4.2.2. dThd(α-6)hdThd

Being the quantitatively most important thymidine adducts formed by the direct effects of gamma radiation, both isomers have been isolated in sufficient quantities for a reasonably complete structural analysis. Despite obtaining a good 20 mg of the first isomer, all attempts to crystallise this product have failed and hence the structural analysis rests on the data obtained from FAB mass spectrometry and especially from extensive 1H and 13C NMR spectroscopy. It should be mentioned that although the presence of two asymmetric carbons implies four possible diastereoisomers, each configuration at C6 induces a preferred configuration at C5, as will be discussed in more detail later.

FAB mass spectrometry shows the molecular weight to be 484 amu, exactly twice that of thymidine. The UV absorption spectrum shows a maximum at 262 nm indicating unsaturation of at least one of the base residues.

The two isomers are distinguished by 1H NMR, where each molecule shows the same group of signals which differ slightly in the coupling constants and chemical shifts. There is an approximately twofold difference in the yields of the two isomers, the most important being
that which is eluted first on reversed phase HPLC. In the following
general discussion on the NMR features of these molecules, data will be
given for the most abundant, the corresponding values for the second
isomer being given in parentheses.

A cursory look at the one dimensional $^1$H NMR spectra (Figures 1.14
and 1.15) shows the presence of only one vinyl proton in the form of a
singlet at 7.69 ppm (7.76 ppm), suggesting saturation of one of the base
residues. We also note only one methyl group resonance which is at too
high a field to be attached to an alkenic carbon and is no doubt that
belonging to the saturated base. This is supported by its multiplicity
which shows it to be coupled to a vicinal proton whose signal appears at
3.10 ppm (3.13 ppm) with a chemical shift which correlates well with
that of the H5 methyne proton of 5,6-dihydrothymidine (2.84 ppm). The
fate of the other methyl group is indicated by the presence of a classic
ABX system. The AB part is seen as two quartets at 2.74 ppm and 2.60 ppm
(2.81 and 2.49 ppm) with a geminal coupling constant of 14.6 Hz for both
isomers. In both cases the vicinal coupling to X is greater for the
upfield CH$_2$ proton. The X group found at 4.07 ppm (4.07 ppm) is assigned
as H6 of the saturated base, and the CH$_2$ group the remains of the methyl
group attached to the unsaturated pyrimidine. The other signals present
are accounted for by two intact 2'-deoxyribose residues. The fact then
that this compound has a saturated base with a hydrogen at C5 and a H6
which is also coupled to a non-equivalent CH$_2$ group which clearly links
the two base residues provides very strong evidence for the structure
given below.
Figure 1.14: 400 MHz $^1$H NMR spectrum in D$_2$O of Isomer I of dThd α-6-Thd.
Figure 1.15: 400 MHz H NMR spectrum in D$_2$O of Isomer II of dThd.
TABLE 1.3
CHEMICAL SHIFTS FOR 5',6-CYCLO-5,6-DIHYDROTHYMIDINES (PPM)

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COUPLING CONSTANTS FOR 5',6-CYCLO-5,6-DIHYDROTHYMIDINES (Hz)

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TABLE 1.4: ¹³C CHEMICAL SHIFTS FOR THYMIDINE ADDUCTS

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a - A = unsaturated residue; B = saturated residue.
b - not determined.
The assignments of all the proton resonances for each isomer were made by two-dimensional correlation spectroscopy. Figure 1.16 shows the corresponding spectrum for the second isomer, the transparency showing the correlations. Unfortunately this experiment does not allow us to assign a set of osidic protons to a particular base since C6 of each base is separated from C1' by N1 which carries no protons. The downfield shifted group of signals were tentatively assigned to the sugar attached to the unsaturated base due to the expected diamagnetic effect. This was ultimately shown to be correct by a NOE difference spectrum where the H6 singlet of the unsaturated base was irradiated. A distinct nuclear Overhauser effect can be seen on the H1', H2' and H3' protons of the downfield shifted sugar. The H1' response was expected, however the H2' and H3' responses give the additional interesting information that effectively the H6 proton must lie above the C2' position in order to give a NOE on all three protons indicating that the molecule has an almost pure anti conformation. A slight signal is also detected for the CH₂ group confirming that the latter is aligned with H6 of the unsaturated base.

A NOE difference spectrum also allowed us to define the relative configurations at C5 and C6 of the saturated base. Irradiation of the methyl group gave a response at H6 showing it to be cis to the methyl group. We therefore know that the two isomers are 5R6R and 5S6S but as yet we are unable to assign a particular configuration to a particular NMR spectrum. The NOE difference spectra carried out on the first isomer are given in Figure 1.17.

The ¹³C NMR spectra of each isomer have also been recorded and the assignments made by two-dimensional ¹H-¹³C heteronuclear correlation spectroscopy (Table I.4).

At 250 MHz the ¹H NMR spectra are somewhat complicated, especially in the upfield region where the H2' signals are superimposed. To verify the attributions of these and other multiplets, the first isomer was analysed by two-dimensional J-resolved spectroscopy (Figure 1.18). Both isomers were analysed at 400 MHz where the picture became somewhat clearer and the assignments finally verified by computer iteration at two fields strengths, 400 MHz and 250 MHz using the LAOCOON III program.
Figure 1.6: 2D COSY spectrum for dThd(α-6)tdThd II.
Figure 1.17: NOE difference spectra of the Isomer I of dThd(α-6)dThd. (a) Saturation of the methyl group resonance. (b) Saturation of the vinyl H6 resonance. (c) Normal spectrum.
Figure 1.18: 250 MHz 2D-J-resolved $^1$H NMR spectrum for Isomer I of dThd(α-6)hdThd in D$_2$O.
The iteration at two field strengths confirms unambiguously the signal assignments and coupling constant measurements.

1.4.2.3. dThd(α-5)hdThd - the spore photoproduct

This is a structural isomer of dThd(α-6)hdThd. Two isomers exist, 5R and 5S, and again assignment of a particular ¹H NMR spectrum to a particular diastereoisomer is not possible at the moment, both isomers having to date resisted all attempts to crystallise them. In the 400 MHz ¹H NMR spectrum (Figures 1.19 and 1.20), we note the presence of only one vinyl proton at 7.83 ppm (7.70 ppm). As for the dThd(α-6)hdThd adduct, two groups of osidic residue protons may be distinguished, the assignments of which have been made by two-dimensional correlation spectroscopy at 200 MHz. The methyl group of the saturated residue takes the form of a singlet at 1.28 ppm (1.16 ppm) and no corresponding H₅ proton is observed at the expected chemical shift region of 3 ppm. The methylene bridge is now shown as a simple AB quartet at a chemical shift similar to that observed for the AB part of the ABX system of dThd(α-6) hdThd. We also note a tight AB quartet centred at 3.38 ppm (3.41 ppm) assigned as the ring CH₂ group at the 6 position. This is good evidence for the presence of a methylene bridge to C5 of the saturated residue. The ¹³C spectrum shows the appearance of second CH₂ group at 46.49 ppm (46.96 ppm) compared to the spectrum obtained for dThd(α-6)hdThd where we find a methyne C6 at 53.05 ppm (55.86 ppm) (Table I.4). The ¹³C resonances were again assigned by heteronuclear ¹H-¹³C correlation spectroscopy.

1.4.2.4. dThd(α-α)dThd

The FAB mass spectrum of this product shows a molecular mass of 482 amu indicating two atomic mass units less than a strict thymidine dimer. The ¹H NMR spectrum (Figure 1.21) shows only one set of nucleoside protons - evidence for a plane of symmetry in the molecule. We detect a vinyl proton at 7.62 ppm hence the 5,6-double bond is likely to be intact. This is supported by an absorbance maximum at 272 nm. The rest of the ¹H NMR spectrum shows the usual signals for a non-derivatised osidic moiety. We detect no methyl group in the molecule, instead we note a two-proton singlet at 2.57 ppm. We therefore have two nucleoside residues which are unsaturated and contain a methylene group instead of
Figure 1.19: 400 MHz 1H NMR spectrum in D2O of Isomer I of the spore photoproduct nucleoside.
Figure 1.20: 400 MHz 1H NMR spectrum in D$_2$O of Isomer II of the spore photoprodct nucleoside.
a methyl group, and a plane of symmetry. This is good evidence for the dThd(α-α)dThd structure.

1.4.2.5. dThd(3-5)hdThd

The structural analyses of the two isomers (5R, 5S) of this product rest heavily on the one and two-dimensional $^1$H NMR spectra, the small amount of product obtained limiting the analytical techniques available. The product is clearly dimeric, a fact confirmed by the FAB mass spectrum giving a molecular mass of 484 amu (exactly twice that of thymidine) and from the $^1$H NMR spectra (Figure 1.22 and 1.23) we may distinguish two complete groups of osidic protons indicating that none of the sugar carbons are involved in the internucleoside bridge. We detect one vinyl proton signal at 8.02 ppm (8.03 ppm) seen as a tight quartet through long-range coupling to the methyl group doublet at 2.02 ppm (2.03 ppm) with a J value of 1 Hz. It is evident from this that the bridging does not involve any part of the vinyl system of the unsaturated base in contrast to the dThd(α-5)hdThd and dThd(α-6)hdThd adducts. The fact that the saturated base methyl group signal takes the form of a singlet would suggest that the thymidyl residue is attached to C5. Its chemical shift of 1.79 ppm (1.76 ppm) is too high for a C-C link (c.f. dThd(α-5)hdThd; $^1$CH$_3$=1.28 ppm) suggesting bonding of a highly electronegative group at C5. The only other non-osidic proton system takes the form of a very wide AB system, one proton having a chemical shift of 3.46 ppm (3.51 ppm), similar to that of the 6-yl protons of the spore photoproduct (3.38 ppm), the other showing a large downfield shift at 4.39 ppm (4.38 ppm).

This was also recently shown to be the case (70) for the corresponding proton signals (3.57 ppm and 4.17 ppm respectively) of the adduct of 4-N-oxyl-2,2,4,4-tetramethylpiperidine, a known radiosensitiser, to the 5 position of thymidine, the structure of which is given in Figure 1.24. We might attribute the downfield shift of one of the C6 protons to
Figure 1.22 : 400 MHz 1H NMR spectrum in D2O of isomer I of 1,1-dimethyl-3-(5-phenylphenyl)methanone.
Figure 1.24: TAN adduct to the 5 position of thymidine (70).

its proximity to the N3 lone pair, or to a greater proximity on average of the ring carbonyl groups, the attached pyrimidine being able to rotate fairly freely.

Were we to propose a hydroxyl group at C5 to explain the downfield shift of the methyl signal, such a severe deshielding of the methyl group cannot be accounted for by a 5-OH group, that of 5-hydroxy-5,6-dihydrothymidine being at 1.47. In addition, we would expect the AB quartet to be much tighter as in the case of the latter reference compound where the literature (55) gives 3.43 ppm and 3.51 ppm respectively for the 5S isomer and two magnetically equivalent protons for the 5R isomer at 3.48 ppm.

The evidence suggests that the linkage between the two nucleoside residues occurs at C5 of a saturated 5,6-dihydrothymid-5-yl residue and a strongly electronegative heteroatom of the unsaturated thymidyl residue, probably situated in the base residue since no perturbation of either sugar residue is evident. Possible candidates for the heteroatom involved in the bridge to C5 of the second nucleoside are N3, O2 and O4. The formation of an oxygen centred radical is possible since the 5,6-dihydrothymid-5-yl radical has a mesomeric oxyl radical structure (Figure 1.25), however the expected structure of the dimer would lack

Figure 1.25
the vinyl proton and would contain a second AB system which is not apparent from the $^1$H NMR spectrum. A radical cation in neutral solution can clearly not deprotonate at the carbonyl groups, and in electron gain centres O4 generally holds the negative charge while the unpaired electron density resides at ring carbons, namely C6. The thymidine radical anion may possibly protonate at O2, although there exists no direct evidence for this, and again the radical is most likely to be centred at the C2 atom. The most likely heteroatom involved is N3, the corresponding radical being readily accounted for by the deprotonation of the radical cation.

Schulte-Frohlinde et al (71) considered the possibility of N3 deprotonation in the laser photoionisation of poly-U, pointing out that the uracil radical cation rapidly deprotonates at N1 but that it is an open question as to whether with the N1-substituted uracil moiety the proton elimination occurs at N3 with such a high rate that it is a dominant process or whether other deprotonation sites are favoured. It seems quite clear that deprotonation of the thymidine radical cation occurs preferentially at the methyl group, however the suggestion of a contribution, although probably a minor one, from an N3 deprotonation is not at all unreasonable. Further support for the N3-C5 bond comes from the $^1$H NMR spectrum of this product in acetone-$d_6$ where we observe only one NH signal (Figure 1.26) instead of the expected two were the

Figure 1.26: NH and H6 signals from the 400 MHz $^1$H NMR spectrum of dThd(3-5)hdThd recorded in DMSO-d6.
nitrogen not to be involved in the internucleoside link. The similarities between the spectral features of this product and the thymidine-TAN adduct support this conclusion. In addition, the downfield chemical shift value of the methyl group at 1.79 ppm may be explained by the proximity of the two imide carbonyl groups or the N3 lone pair as already mentioned.

1.4.2.6. dThd(5'-6)hdThd.

Two isomers have been obtained for this product. They have distinctly different conformational properties as discussed later, but they show the same general $^1$H NMR features. The 400 MHz spectrum of each isomer are shown in Figures 1.27 and 1.28. The connectivities of the various proton signals were made in the case of the first isomer by 400 MHz two-dimensional correlation spectroscopy.

The spectrum of each isomer has a single vinyl proton signal at 7.92 ppm (7.45 ppm) seen as a tight quartet through coupling to the methyl group at 1.90 ppm (1.92 ppm). We detect also a methyl signal upfield in the form of a doublet through coupling to the H5 proton whose signal is observed at 3.11 ppm (3.22 ppm). The above analysis provides good evidence for the presence of one saturated and one unsaturated thyminyl residue.

The evidence for the site of internucleoside bridging comes from the analysis of the sugar moiety protons. The presence of a C5 proton and only one H6 signal at relatively low field in the 4 ppm region points to a bridge at the C6 position of the saturated base residue. We note one complete set of furanose protons, however it is evident that the other nucleoside lacks one of the geminal protons of the ABX system that usually constitutes the exocyclic hydroxymethyl group. The H5' proton gives rise to a pseudotriplet for the first isomer and a double doublet for the second at 4.29 and 3.82 ppm respectively. The two-dimensional correlation spectrum shows this proton to be coupled to a proton whose signal appears at 3.82 ppm (3.98 ppm) which is itself coupled to the H5 proton and which must therefore be designated as the H6 proton. The H5' signal also shows coupling in each case to the H4' signals at 4.04 and 3.93 ppm respectively. This suggests strongly that
Figure 1.27: 400 MHz $^1$H NMR spectrum in D$_2$O of Isomer I of dThd(3'-6')dThd.
Figure 1.28: 400 MHz H NMR spectrum in D$_2$O of Isomer II of DTPA(5'-g)-gTPA dm.
The internucleoside link occurs from the C5' carbon of the unsaturated nucleoside residue to the C6 carbon of the saturated one.

The H5H6 coupling constants for each isomer give some indication of the relative configurations at these centres. For the first isomer, the coupling constant is very small at only 1.2 Hz and suggests strongly an equatorial-equatorial relationship. For the second isomer, this coupling is much stronger at 6.4 Hz and is most likely to indicate an axial-equatorial situation.

1.4.2.7. dThd (α-3)dThd.

The structural assignment of this compound has not yet been made unambiguously. The major portion of the structural data suggests the structure given below.

![Structural diagram of dThd (α-3)dThd]

We note from the $^1$H NMR spectrum (Figure 1.29) that the molecule contains two vinyl protons. That giving a signal at 7.58 ppm presents a tight quartet which a 2D correlation spectrum shows to be coupled to the methyl doublet at 1.84 ppm. This indicates an intact 5,6-double bond system of thymidine, the bridge to the second nucleoside residue taking place elsewhere in the molecule. A well-resolved spectrum indicates that the second vinyl proton signal (7.97 ppm) appears as a pseudotriplet being coupled to an AB system (4.35 ppm and 4.38 ppm). This may be interpreted as a thymidyl residue forming a CH$_2$ bridge to the other nucleoside. We note that the chemical shift of this AB system is well downfield of that observed for the same system of the spore photoproduct and the dThd(α-6)hdThd adducts situated around 2.5-3.0 ppm suggesting that the methylene bridge occurs to an electronegative group in the
Figure 1.29: 400 MHz $^1$H NMR spectrum in D$_2$O of dThd(α-3)dThd.
other nucleosidic residue. The signals corresponding to the non-exchangeable furanose protons have the usual chemical shifts and coupling patterns observed for non-derivatised nucleosides and we therefore assume that bridging does not occur to the osidic moiety of either residue. Since both sugar residues and both 5,6-double bonds are intact we must assume bridging via one thymidyl methylene group to a site in the base of the other nucleoside but not at either C5 or C6. We are reduced to consider the C2, C4 or N3 positions.

We assumed firstly that the most likely candidate was N3 since, as discussed above, we have evidence of a deprotonation at the N3 position of a radical cation to form an N3 centred radical. A C2 centred radical is also possible if we accept the possibility of the protonation of a thymidine radical anion at the C2 carbonyl oxygen, for which no ESR evidence has yet been obtained. Two experiments were envisaged to distinguish between these two possibilities. Firstly the $^1$H NMR spectrum of this product in an aprotic solvent should indicate the number of NH groups in the molecule. If the $\text{CH}_2$-$\text{N3}$ linkage exists, we would only expect to observe one NH proton at the N3 position of the other nucleoside residue. The $^1$H NMR spectrum recorded in acetone-$d_6$ indicates two broad signals in the chemical shift region of amino protons apparently denying this possibility.

Secondly, for an N3 link, we would expect a molecular mass of 482 amu, that is two atomic mass units less than a strict thymidine dimer. A C2, or possibly a C4 linkage would tend to give a molecular mass of 484 amu, or 476 amu if dehydration occurs. The FAB mass spectrum of this product shows a molecular mass of 482 amu, supporting the proposal of an N3 linkage. (In addition, a count of the proton integrals in the $^1$H NMR spectrum indicate 21 non-exchangeable protons instead of the 22 expected for a strict thymidine dimer).

In view of the small amount of product at our disposal, and the difficulties encountered when working with aprotic solvents in dilute solution, we will rest with our suggestion of the dThd($\alpha-3$)dThd structure until we are able to obtain the product in greater quantities in order to continue the analysis further. Our reason for keeping to the proposed structure lies mainly in the fact that we can envisage no other structure involving the C2 and C4 positions which may give rise to the
observed molecular mass. Although mass spectral data may from time to time be unreliable, the molecular mass was checked thoroughly and the spectrum run twice in order to rule out artifacts as far as possible. Since the presence of four nitrogen atoms in the molecule necessitates an even mass, we would have to be out in our calculation by two atomic mass units, which is unlikely. In addition the mass spectra of the dThd(3-5)hdThd adducts were run at the same time and gave the expected mass of 484 amu.

1.5. Mechanistic studies of the degradation of thymidine via the direct effects

Introduction

In this section we shall describe experiments performed with the aim of clarifying the mechanisms involved in the formation of the products isolated in the study of the direct effects of gamma radiation on thymidine.

The experiments chosen all involve heavy isotopes, the use of which have always served as powerful probes for the elucidation of reaction mechanisms. As will be discussed in more detail below, irradiations in D$_2$O solution and of specifically deuterated thymidine have been carried out in order to determine the sites of hydrogen or proton transfer processes, and the role of molecular oxygen in the formation of certain oxidised derivatives tested by use of $^{18}$O$_2$. Finally, the implication of particular primary charged radical species in the formation of certain quantitatively important products has been demonstrated by quantitation of these when thymidine is irradiated in the presence of various co-solutes.

1.5.1. The gamma irradiation of thymidine in frozen D$_2$O solution

During the formation of various products, both monomeric and dimeric, addition of hydrogen, either as an atom or via electron transfer followed by protonation, to a radical intermediate represents an important step in the saturation of the 5,6-double bond of a thymine
residue. Under the initial experimental conditions, it was impossible to deduce the donor implicated in the various reduction steps. Hydrogen addition may occur in two distinct ways. Firstly, as a protonation of a pristine radical anion at C6 in the cases of 5,6-dihydrothymidine, 5,6-dihydrothymine and 5-hydroxy-5,6-dihydrothymidine formation, and as hydrogen atom addition to an uncharged radical, at C5 in the cases of 5,6-dihydrothymidine, 5,6-dihydrothymine, 5',6-cyclo-5,6-dihydrothymidine and 6-hydroxy-5,6-dihydrothymidine, and at C4' in the epimerisation process leading to formation of the α-lyxo form of thymidine. Addition to C5 is also likely to occur in the formation of various dimeric products, in particular dThd(α-6)hdThd (in the case of a consecutive mechanism) and dThd(5'-6)hdThd.

We may classify the possible donor sites into two groups:

a) exchangeable protons either in the form of hydration water or OH and NH functions on the substrate molecules or

b) non-exchangeable aliphatic substrate protons.

In order to demonstrate the first possibility, and consequently rule out the second, we irradiated thymidine in frozen D₂O solution under conditions of temperature and concentration identical to those of the previous experiments. For reasons of time, the sample was only irradiated sufficiently to give workable yields of the quantitatively most important products, the limiting factor being the necessity to obtain sufficient quantities of the dThd(α-6)hdThd adducts in order to answer the important mechanistic question of whether the process involved in their formation is consecutive, with distinct radical intermediates, or concerted involving a possible hydrogen transfer from the methyl group of one thymine residue to the C5 position of the ultimately saturated base.

The following products were isolated and analysed by 400 MHz ¹H NMR spectroscopy.

1. 5,6-dihydrothymidine
2. 5,6-dihydrothymine
3. 5-hydroxy-5,6-dihydrothymidine
4. 6-hydroxy-5,6-dihydrothymidine
5. 5',6-cyclo-5,6-dihydrothymidine
6. dThd(α-6)hdThd
All products whose proposed mechanism of formation involved an initial protonation of the thymidine radical anion were shown by $^1$H NMR to have a deuterium atom incorporated at C6 at effectively 100%. It was interesting to observe that for both 5,6-dihydrothymidine and its base analogue, addition occurs with only a slight stereoselectivity (approx. 60:40), with a slight preference for addition to the position responsible for the downfield H6 proton NMR signal. We might effectively predict that proton transfer occurs from the region of highest electron (spin) density resulting in deshielding of the proton once transferred compared to that of the geminal proton, since hydrogen bonding to the group responsible for this deshielding, probably the hydroxymethyl group at C4' or the ring O1' oxygen of the furanose moiety, might position a hydration water molecule, or at least increase its likelihood of being positioned on the appropriate side of the base ring plane. Alternatively, direct transfer of the C5' hydroxyl deuterium atom cannot be ruled out. It should be mentioned that thymidine has been shown to adopt the anti conformation (72).

All products which are formed via the intermediate formation of a 5-yl radical also show incorporation of deuterium at this position when thymidine is irradiated in frozen D$_2$O solution. These compounds include 5,6-dihydrothymidine, 5,6-dihydrothymine, 5',6-cyclo-5,6-dihydrothymidine and 6-hydroxy-5,6-dihydrothymidine. The $^1$H NMR spectra all show a lack of the one proton multiplet at the expected chemical shifts, the methyl signal collapses from the usual doublet to a singlet and the H6 proton signal shows the disappearance of the expected coupling to H5.

The fact that hydration water should play such an important role in thymidine degradation during irradiation in frozen aqueous solutions is not surprising. Mathur-De Vré et al (48), as mentioned earlier, demonstrated the presence of mobile hydration water at temperatures as low as 196K in frozen DNA solutions and we might expect the same phenomenon at the nucleoside level.

It is true that these results might support the occurrence of the indirect effects of the gamma radiation since they would also be expected to lead to incorporation of deuterium into the 5,6-double bond. However, we would nevertheless also expect a considerable contribution to the yields of these products from the direct effects as discussed earlier, and the fact that deuterium incorporation always occurred at
almost 100 % suggests strongly that during direct effect processes, hydration water plays an important role in proton and hydrogen donation. We might also confidently propose this as the source of the hydroxyl anion to C6 of the thymidine radical cation.

We now come to the evidence provided by this experiment on the mechanism involved in the formation of the dThd(α-6)dThd biadducts. The signals in Figure 1.30 show that even during irradiation in D2O solution, there remains a considerable incorporation of 1H (approx. 50 %) at C5.

![H5 and H2 signals for dThd(α-6)dThd](image)

**Figure 1.30** : H5 and 2" NMR signals for dThd(α-6)dThd isolated from irradiation of thymidine in D2O

The evidence that some deuterium has also been incorporated may be explained by a contribution from a consecutive mechanism, but also by slow exchange with solution D2O during annealing and, after purification, preparation of the sample for 1H NMR analysis. In addition, we have shown that only two of the possible four diastereoisomers can be isolated. It is highly probable that all four are formed during irradiation, although a preference might exist for those found, and therefore on annealing the irradiated sample, a rapid conversion in D2O may occur resulting in incorporation of deuterium at C5 for all molecules formed with an initial 5R6S or 5S6R configuration. Whatever this contribution, we may clearly say that 50 % of the product is formed by addition of a hydrogen as opposed to a deuterium atom to the C5 position. This figure is almost certainly an underestimation of the true value. Furthermore, the result that the 5,6-dihydrothymidines showed a 100 % incorporation of deuterium at C5, and the fact that we could see no reason why the two intermediate 5-yl radicals should behave differently, strongly suggests that the major process does not involve transfer of an exchangeable proton to C5.
1.5.2. Gamma irradiation of thymidine-CD₃

We have tested the hypothesis that the dThd(α-6)hdThd adducts are formed via a concerted process and that transfer of a methyl hydrogen occurs to the C5 position of the eventual saturated residue. To do this we synthesised thymidine completely deuterated at the methyl group.

![Diagram of thymidine synthesis](image)

*Figure 1.31*: The synthesis of CD₃-thymidine.

The synthesis involved a two step process (Figure 1.31). The first step comprised the synthesis of 5-hydroxymethyl-2'-deoxyuridine deuterated at the hydroxymethyl group according to the procedure of Baker et al (59) using deuterated paraformaldehyde, with a slight modification. According to the published methodology, the reaction mixture should be maintained at 65° for four days whereupon a yield of 60% is obtained. Contrary to the reported heat instability of 5HMdUrd (73), we found that heating the reaction mixture under reflux did not produce detectable decomposition of the product and afforded yields in excess of 90% after only two hours.

The second step involved the catalytic reduction of the hydroxymethyl group to a methyl group using a Rhodium/alumina catalyst in the presence of deuterium gas. To our knowledge, this is the first time this methodology has been used for the preparation of deuterated thymidine. The usual reaction medium is 50% acetic acid. For the synthesis of the deuterated product, we suspected, and indeed found it to be the case, that during hydrogenation, the acid proton is also involved and the thymidine produced in the H₂O/CH₃COOH medium was shown by ¹H NMR to contain on average half a proton at the methyl group. The problem was resolved by preparing a deuterated solvent by leaving D₂O
and acetic anhydride to stand in the appropriate proportions to yield 50:50 D$_2$O/CH$_3$COOD. Under these conditions, catalytic hydrogenation leads to formation of a methyl group deuterated at 100%. Purification of the 5HMDUrd is not necessary since final product purification is carried out by reversed phase HPLC. Isolation of the 5HMDUrd from the 2'-deoxyuridine starting material is not possible on reversed phase but is efficiently purified on silicagel using Solvent II.

The CD$_3$-thymidine was irradiated under the usual conditions in a frozen H$_2$O solution.

The dThd($\alpha$-6)hdThd adducts were isolated by reversed phase HPLC and submitted to FAB mass spectrometry and 400 MHz $^1$H NMR spectroscopy. From the mass spectrum, it is clear that the molecule has the molecular mass of a dimer of thymidine plus five atomic mass units (M+Na=512 a.m.u.). The peak corresponding to M+Na+1 has an abundance 30% that of M+Na, 23% of which is expected from natural abundances of heavy isotopes. In view of the high background noise, and the fact that the spectrometer was controlled on a non-deuterated nucleoside showing also a higher than expected M+Na+1 peak, it would appear that there is no incorporation of deuterium at C5. Conclusive confirmation of this is

**Figure 1.32**

H5 and H6 regions of the 400 MHz $^1$H NMR spectrum in D$_2$O of dThd($\alpha$-6)hdThd from (a) irradiation of CD$_3$-thymidine and (b) from the gamma irradiation of thymidine.

yielded by the 400 MHz $^1$H NMR spectrum. Figure 1.32 shows the H6 and H5 regions of the normal and deuterated samples of the first isomer of
dThd(α-6)hdThd. The H6 doublet (the AB part of the ABX system comprises two deuterium nuclei) shows a clear coupling with the H5 proton with no singlet contribution whatsoever. The results show that the mechanism involved in the formation of the dThd(α-6)hdThd adducts is not concerted and that no transfer of a methyl proton occurs to C5.

1.5.3. **Gamma irradiation of thymidine in the presence of $^{18}$O$_2$**

In aerated aqueous solution, the formation of 5-hydroxy-5,6-dihydrothymidine and the thymidine diols occur via the formation of an initial 5-yl radical with subsequent addition of molecular oxygen to give a peroxy radical which, after reduction and degradation of the resulting hydroperoxide, yields the corresponding alcohol. The role of oxygen is also evoked in the formation of 5-hydroxymethyl-2'-deoxyuridine and 2-deoxyribonolactone with additions to 5-methyl and C1' centred radicals respectively. In addition, it has been shown (74) that oxygen adds to 5-yl radicals in frozen aqueous samples. In order to test this hypothesis, we saturated the thymidine solution with $^{18}$O$_2$ before freezing and irradiated the sample subsequently under the usual conditions. Care was taken in the preparation of the sample to exclude all $^{16}$O$_2$ before saturation with $^{18}$O$_2$, which was of an isotopic purity of 99.8%, by successive degassing stages under vacuum before allowing entry into the flask of the latter oxygen only. Isolation of the products was done in the usual way, and the samples analysed by FAB mass spectrometry. From Figures 1.33 and 1.34 we can see that incorporation of $^{18}$O has occurred for 5-hydroxy-5,6-dihydrothymidine and the thymidine diols but not at 100%. In fact in both cases the heavier isotope accounts for only 30% of the total yield of each product. We may interpret this result in two ways. Either the saturation of the solution of thymidine with $^{18}$O$_2$ was inefficient and there remained a considerable amount of normal oxygen of atomospheric origin, which considering the care taken to avoid this eventuality cannot, in our view, account for an almost 70% oxygen-16 content, or the mechanism proposed far from accounts for the major portion of the yields observed. Clearly the other possible mechanism involves the indirect-effects of the gamma radiation.
Figure 1.33: FAB mass spectrum of 5,6-dihydroxy-5,6-dihydrothymidine from the gamma irradiation of thymidine in the presence of $^{18}O_2$.

Figure 1.34: FAB mass spectrum of 5-hydroxy-5,6-dihydrothymidine from thymidine irradiated in the presence of $^{18}O_2$. 
Further information may be gleaned from the mass spectrum of the thymidine diol to clarify this point. If the indirect effects are indeed the major processes leading to the formation of these compounds, we would expect a distribution of the $^{18}O$ label between the C5 and C6 positions, since hydroxyl radical addition to the 5,6-double bond of thymidine is known to occur at both sites with a preference for C5. Close inspection of the fragment masses compared to those of a non-labeled sample will indicate whether the $^{18}O$ is situated at one, the other or both positions. For the diol, the primary mass loss is dehydration, the fragment retaining the oxygen at C5. The observation of a significant peak at m/z 461 indicates oxygen labelling at this site. Unfortunately, it is difficult to demonstrate oxygen label at C6, although we feel that the label occurs primarily at C5. Since the hydroxyl radical adds preferentially to C5 under the indirect effects, diol formation may occur in this case via oxidation of the reducing 6-yl radical to form the carbonium ion which may then add water.

1.5.4. The direct effects of gamma rays on thymidine bromohydrin

A complicating factor in the assignment of a particular mechanism to the formation of a degradation product is the large number of initial and intermediate radical species possible, several pathways being able to account for the formation of any one product. In order to test whether a particular radical intermediate is implied in the formation of one or more of the observed products, we need ultimately to be able to produce this radical specifically under identical experimental conditions, and follow its degradation during annealing. In a recent collaborative study (74), it was shown by ESR that the irradiation of 5-bromo-6-hydroxy-5,6-dihydrothymidine yields radical II. It was also shown that in the presence of molecular oxygen, this radical is efficiently converted to the corresponding peroxo radical (Figure 1.35).

![Figure 1.35](image-url)
One recalls that this radical and its oxygen adduct are proposed intermediates in the formation of the thymidine "hydrates" and the thymidine diols respectively.

The two major products isolated were thymidine and 5-hydroxymethyl-2'-deoxyuridine. The formation of the thymidine diols via a radiolytic process cannot unfortunately be confirmed since, although present in the irradiation mixture in large quantities, this compound is formed by the fairly rapid hydrolysis of thymidine bromohydrin in aqueous solution. Nevertheless, taking into account the ESR data mentioned above, these are likely radiolysis products in the presence of traces of oxygen.

A surprising result is the complete absence of the thymidine "hydrates". If the 6-hydroxy-5,6-dihydrothymid-5-yl radical is not involved in the formation of 6-hydroxy-5,6-dihydrothymidine, it is difficult to propose a possible alternative to explain its formation by direct or indirect effect processes occurring during the gamma radiolysis of thymidine in frozen aqueous solution. One possible explanation might be the need for the presence of reducing species if electron transfer to the 5-yl radical is a critical step in the formation of these products. It is true that the thymidine "hydrates" are known to be unstable and dehydrate to give thymidine. Coincidentally, thymidine is the major product of the radiolysis of the bromohydrin. However, two important comments have to be made before we attempt to neatly explain the absence of the "hydrates" and the presence of thymidine by dehydration. Firstly, in the gamma radiolysis of thymidine, the "hydrates" were isolated in important quantities even though the separation was longer owing to the complexity of the mixture, and the treatment the mixture was subjected to was far more vigorous, involving heating to 100° during the precipitation of the undegraded thymidine. Secondly, an experiment was carried out where the irradiated bromohydrin mixture was injected onto a reverse phase preparative HPLC column immediately upon annealing in order to isolate the thymidine from the possible presence of the hydrates and it was shown that thymidine was formed in considerable quantities by radiolytic processes. Unfortunately, we did not check whether thymidine could be observed to be formed in the fraction expected to contain the "hydrates". Finally, it is not possible to rule out the formation of the "hydrates" completely since the liberation of hydrobromic acid during the irradiation and hydrolytic degradation of
the bromohydrin might considerably increase the rate of dehydration via an E2 mechanism compared to the thymidine irradiation experiments where the mixture remained at close to neutral pH throughout the separation process.

The most likely explanation for the formation of thymidine through radiolytic processes is by the occurrence of a reversible loss of hydroxide anion at C6 leading to the thymidine radical cation, its proposed precursor. Simple capture of an electron to regenerate thymidine is not an unlikely process (Figure 1.36).

![Figure 1.36](image-url)

Figure 1.36
Possible mechanism for the formation of thymidine from the gamma radiolysis of thymidine bromohydrin.

We now come to the formation of 5-hydroxymethyl-2'-deoxyuridine (5MdUrd) in quantities similar to those of thymidine. It was initially thought that the radical cation formed as described above may deprotonate to give a methylene radical which after addition of molecular oxygen may yield the desired product. There is some evidence to suggest the formation of a methylene radical since it has been recently been shown by Méleard et al (75) that when thymidine bromohydrin is irradiated in deaerated aqueous solution at ambient temperature, 5-thymidyl-6-hydroxy-5,6-dihydrothymidine was found to be formed. It was proposed that this product is the result of the combination of a methylene radical with a 6-hydroxy-5,6-dihydrothymid-5-yl radical. However, when irradiated in the presence of $^{18}_0$, thymidine bromohydrin yields $^{18}$HMdUrd which shows no incorporation of $^{18}_0$ at the hydroxymethyl group, as was
shown to be the case when thymidine was used as the substrate.

We may account for the formation of both thymidine and 5HMdUrd if we assume the formation of the thymidyl radical to be an important process. Bernhard (76) suggested the occurrence of the following charge transfer process between an adjacent pair of thyminyl radicals.

\[
\begin{align*}
2 \cdot \text{H} & \rightarrow \text{H} + \text{H}
\end{align*}
\]

The carbanion might be expected to protonate to give thymidine and the associated carbonium ion to hydrate yielding 5HMdUrd. To test this hypothesis, thymidine bromohydrin was irradiated in frozen D$_2$O solution and the thymidine isolated chromatographically. The $^1$H NMR spectrum showed a three proton methyl signal and the aromatic H6 signal presented a clean tight quartet verifying an intact methyl group. As far as thymidine is concerned then we may fairly confidently rule out the participation of the thymidyl radical in its formation. To explain the formation of this product from the 6-hydroxy-5,6-dihydrothymid-5-yl radical is difficult. We might propose instead the bromohydrin radical cation described by Gregoli et al (74) as the radical precursor.

It is also surprising to observe no formation of biadducts resulting from the direct combination of the 5-yl radicals as was shown to be the case for room temperature experiments. The formation of the dimers having the structures shown below are known to be induced by irradiation of thymidine bromohydrin in deaerated aqueous solution. We might expect the increased stability of the radicals at low temperatures and the increased proximity of substrate residues due to base stacking to increase the probability of radical combination. It is clear that
temperature would appear to play an important role in the behaviour of these radicals.

\[
\begin{align*}
&\text{\begin{center}
\includegraphics[width=\textwidth]{image.png}
\end{center}}
\end{align*}
\]

1.5.5. A quantitative analysis of thymidine degradation in frozen aqueous solution: a study of the effect of electron scavengers, radiosensitisers and nucleoside co-solutes

The primary events in the degradation process under the direct effects of the incident ionising radiation are the removal and capture of electrons to form charged radical species (77). These species then interact with their environment in such a way as to form stable diamagnetic products. It is clear that any constraint we apply to the system having the effect of reducing the yield of a particular primary charged radical will have a concommitant effect on the yield of its stable progeny. We therefore have a means of linking the initial charged radicals with the final diamagnetic products providing support for the proposed mechanisms of formation.

The methodology involved irradiating $^{14}\text{CH}_3$-thymidine in the presence of various co-solutes, effecting a separation of the products by two-dimensional thin layer silicagel chromatography, assignment of the autoradiography spots by co-chromatography and scintillation counting of the activity eluted from each. Not all products were quantitated since the complexity of the mixture lead to overlap of many products on the t.l.c. plates. Fortunately, those products most likely to show a clear response to the presence of the co-solute were well separated by the two solvent systems chosen.
The results of this study are shown in Table I.5.

1.5.5.1. *N*-iodoacetamide

*N*-iodoacetamide is a well known radical scavenger. Gregoli et al (47) showed it to efficiently quench thymidine radical anions in gamma irradiated dTMP. They also showed (78) that in the presence of *N*-iodoacetamide, the yield of OH$^-$ addition radicals to 2'-deoxyadenosine-5'-monophosphate (dAMP) increased. This was explained by a competition for the dissociated electron by the scavenger at once reducing the number of adenine radical anions and consequently their H-adducts and, by interrupting the neutralisation processes among A$^-$ and A$^+$ via electron transfer through overlapping orbitals of the same stack, increasing the observed yield of radical cations. In the present study we would expect the presence of an electron scavenger to reduce the yields of all products derived from the thymidine radical anion. Inspection of Table I.5 shows that the presence of *N*-iodoacetamide results in a 60% reduction in the yields of the two diastereoisomers of 5,6-dihydrothymidine confirming the role of the radical anion in its formation. A similar reduction (42%) is observed in the yield of 5,6-dihydrothymine. This result has important implications for the mechanism of formation of this product. It is clear that the precursor leading to the formation of 5,6-dihydrothymidine and its base analogue is the same, that is the radical anion which undergoes protonation at C6. The high yield of 5,6-dihydrothymine observed cannot be explained by the reduction of radiation induced thymine. There would appear to be a link between saturation of the base and release of the furanose residue. It might be thought that the 5-yl radical may possibly abstract a proton in the sugar moiety. This was proposed to happen during the gamma irradiation of poly-U by Schulte-Frohlinde (79), and in frozen aqueous DNA solution by Cullis and Symons (80) to explain the induction of chain breaks by base centred radicals. However, this abstraction would occur at one sugar down the chain to that to which the base radical is attached. This is not the present case and the fact that irradiation in frozen D$_2$O solution showed the 5-yl proton to originate from hydration water or exchangeable substrate hydrogens would seem to rule out this possibility. This problem remains to be resolved.
<table>
<thead>
<tr>
<th>Compound</th>
<th>dThd</th>
<th>dThd + dThd</th>
<th>dThd + Ac</th>
<th>dThd + 5-Nitro-2-furoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cis-anti(-)</td>
<td>0.029</td>
<td>0.039</td>
<td>0.016</td>
<td>0.029</td>
</tr>
<tr>
<td>Cis-anti(+)</td>
<td>0.025</td>
<td>0.043</td>
<td>0.043</td>
<td>0.025</td>
</tr>
<tr>
<td>Cis-Syn</td>
<td>0.111</td>
<td>0.463</td>
<td>0.304</td>
<td>0.054</td>
</tr>
<tr>
<td>5R(+)-5,6-dihydrothymidine</td>
<td>0.145</td>
<td>0.353</td>
<td>0.120</td>
<td>0.077</td>
</tr>
<tr>
<td>5S(-)-5,6-dihydrothymidine</td>
<td>0.144</td>
<td>0.330</td>
<td>0.084</td>
<td>0.060</td>
</tr>
<tr>
<td>5-hydroxymethyl-2'-deoxyuridine</td>
<td>0.277</td>
<td>0.324</td>
<td>0.166</td>
<td>0.055</td>
</tr>
<tr>
<td>Thymine</td>
<td>0.277</td>
<td>0.324</td>
<td>0.166</td>
<td>0.055</td>
</tr>
<tr>
<td>5,6-dihydrothymine</td>
<td>0.084</td>
<td>0.330</td>
<td>0.144</td>
<td>0.043</td>
</tr>
<tr>
<td>Overall</td>
<td>1.470</td>
<td>2.416</td>
<td>1.746</td>
<td>1.156</td>
</tr>
</tbody>
</table>

b not determined

a dose = 3.6 x 10^4 Gy
It is expected that there be an enhancement of the yield of the radical cation since the scavenger would tend to prevent the return of a dissociated electron. This is confirmed by the observed important increase in the yield of 5-hydroxymethyl-2'-deoxyuridine (56 %). This result would appear to support the association of the thymidine radical cation, although it cannot verify that the mechanism involves the likely deprotonation at the methyl group.

1.5.5.2. 5-Nitrofuroic acid

5-Nitrofuroic acid is a known radiosensitiser (81) and its presence as a co-solute in the irradiation of thymidine might be expected to have an effect on the yield of one or more radiation-induced degradation products. In aqueous solution at ambient temperature, the yield of the diols is greatly increased, and that of dimeric products reduced. Under direct effect conditions, a distinct decrease in the yields of 5,6-dihydrothymidine (29 %) and 5,6-dihydrothymine (70 %) are observed. In this case, not unexpectedly 5-nitrofuroic acid would appear to act as an electron scavenger resulting in a decrease in the yield of the thymidine radical anion. Its role as an in vivo radiosensitiser seems to act in a way other than an enhancement of direct effect damage, although the situation may be different with respect to the purine bases or the sugar-phosphate backbone. It may also radiosensitise by chemical reaction with the DNA or some other cellular target, or by enhancing the indirect effects of the incident radiation.

1.5.5.3. The effect of other nucleosides

The nucleobases have been shown to have different electron affinities. Gregoli et al (82) showed that the electron affinities increased in the order G<A<T<C. In an irradiated mixture of thymidine and 2'-deoxyadenosine we would expect positive hole migration towards the purine and electron migration towards thymidine (82):

\[
\begin{align*}
T^+ + A & \quad T + A^+ \quad \text{(positive hole migration - short range)} \\
A^- + T & \quad T^- + A \quad \text{(electron migration - long range)}
\end{align*}
\]

The increased yield of the thymidine radical anion should be reflected in a corresponding increase in the yield of 5,6-dihydrothymi-
dine and the analogous saturated base. This is indeed observed to be the case with enhancements of 48% and 130% respectively.

We would also expect a decrease in the yield of the thymidine radical cation and we would expect a corresponding decrease in the yield of 5HMDUrd. The observed 10% increase is probably not significant within the limits of experimental error. This result may reflect the fact that positive hole transfer is a short range process in contrast to electron transfer.

According to the order of electron affinities established for the nucleotides (82), we would expect 2'-deoxycytidine to be an electron scavenger with respect to thymidine. However, Table I.5 shows a slight increase (15%) in the yield of the 5,6-dihydrothymidines suggesting that under the present conditions the order of electron affinities for the bases is inverted. We note this time a distinct decrease in the yield of 5HMDUrd (29%) suggesting that perhaps positive hole transfer to cytosine is more efficient than for adenine, although we would expect stacking between thymine and its complementary base to be tighter. The value observed for the dThd-dAdo mixture should be verified. The enhancement of the yield of the thymidine radical anion is not as marked as that observed for the dThd-dAdo mixture suggesting that the difference in electron affinities is not as great as for the latter case. Numerous studies have shown that in irradiated DNA, although electron abstraction is initially indiscriminate, rapid charge migration occurs to yield T^- and G^+ centres only. What is surprising is the difference in the apparent order of electron affinities observed between these and the present study, and that suggested by the conclusive and convincing evidence presented by Gregoli et al (82). We might propose that a difference in the way in which the bases stack in nucleoside and nucleotide aggregates and in DNA may affect this order.

1.6. The mechanisms involved in thymidine degradation under the direct effects

In this section we shall summarise the results obtained and attempt to rationalise the formation of particular derivatives by proposing reasonable mechanisms, processes which do not contradict what is already
known on the direct effects and which, on the contrary, support previous findings concerning the fates of the primary radicals formed. This discussion will be simplified by an initial classification of the products according to the type of mechanism involved or the paramagnetic precursor associated with their formation.

1.6.1. The thymidine radical cation

The loss of an electron from thymidine or any pyrimidine base results in a vacancy in the highest occupied molecular orbital (HOMO) of the π-system and thus are frequently described as π-cations. The positive charge and unpaired electron density will be distributed throughout the pyrimidine ring through resonance processes. The radical cation may be quenched, i.e. returned to its original charge state in a variety of ways in some cases restoring the base to its original structure, in others by interaction with neighbouring molecules yielding stable derivatives. It is clearly the latter type of process which is of primary interest.

1.6.1.1. 6-Hydroxy-5,6-dihydrothymidine

As discussed in Section 1.2.2, anion addition to the pyrimidine radical cation may restore the original charge state of the molecule. Theoretically this addition will occur at the position of highest positive charge density. The addition of OH⁻ to the thymine radical cation was first demonstrated by Sevilla and Engelhardt (54). The resulting 6-hydroxy-5,6-dihydrothymid-5-yl radical must then gain a hydrogen atom to give the stable hydrate. The most likely source of the hydroxide anion is the hydration water and we have shown that the proton which adds to C5 is also an exchangeable one, probably also from hydration water (vide infra). The addition of a hydrogen atom is likely to occur in two steps involving electron transfer to the oxidising 5-yl radical followed by protonation via hydration water.

The isolation of all four isomers of the thymidine "hydrate" does not necessarily indicate non-stereospecificity of the formation of these products although this is likely to be the case in view of the consecutive nature of the mechanism involved. Regardless of the initial
configuration of the hydrate, keto-enol tautomerism at C5 may racemise the configuration at this position, and saturated pyrimidines containing a hydroxyl group at the C6 position are known to ring open and re-close with again epimerisation. Hence any one stereoisomer may give a mixture of all possible structures if left in aqueous solution. This said however we would expect all four to be formed directly. Addition of the hydroxide anion at C6 of the planar \( \pi \)-system should occur from either side of the base ring assuming an even distribution of the donor water molecule around this position throughout the sample. Similarly, protonation at C5 would be expected to be non-stereospecific.

1.6.1.2. 5,6-dihydroxy-5,6-dihydrothymidine

The formation of the thymidine diols also involves hydroxyl ion addition to the thymidine radical cation at C6. There are two possible sources for the incorporation of a hydroxyl group at C5. Firstly, molecular oxygen may add to the resulting 5-yl radical with subsequent reduction and degradation of the peroxy radical. Alternatively a local indirect effect with addition of a hydroxyl radical may also occur. The contributions of each mechanism were tested by gamma irradiation of thymidine in the presence of oxygen enriched in \( ^{18}O_2 \). Incorporation of the heavy isotope at position C5 was shown to have occurred by FAB mass spectrometry, although only at about 30 %, suggesting the involvement of both processes. Since the labelled molecules appear to contain the label at C5, the indirect effect mechanism may involve oxidation of the 6-yl radical preferentially formed with subsequent addition of water to the resulting carbonium ion. A direct effect mechanism may also result in no incorporation of oxygen. Although 5-yl radicals are oxidising, the presence of a radical with a greater oxidising potential may remove an electron to give this time a 5-yl carbonium ion which may interact with water.

All four isomers were isolated. It has been shown that in aqueous solution ring opening occurs at C6 yielding an equilibrium mixture of the cis and trans isomers. Once the configuration at the C6 position has been established according to the non-stereospecific addition of the hydroxide ion, the oxygen will add preferentially trans to the hydroxyl group. Owing to the ring opening reaction mentioned above, all four isomers will subsequently be observed. Once the conformation at C5 is
set, epimerisation cannot occur at this position, and therefore the isomers exist as pairs unlike the "hydrates" where any one isomer may give rise to the other three.

1.6.1.3. 5-hydroxymethyl-2'-deoxyuridine

The mechanism involved in the formation of 5-hydroxymethyl-2'-deoxyuridine (5HMdUrd) in the present study is still not clear. Our immediate thoughts centred on a mechanism similar to that leading to its formation in aerated aqueous solution where a 5-methyl radical, in this case formed by deprotonation of a thymidine radical cation at the methyl group, reacts with oxygen to give the corresponding peroxy radical and, after decomposition, 5HMdUrd. However, the irradiation of thymidine in the presence of 18-oxygen does not lead to incorporation of 18-oxygen at the hydroxymethyl group, and we may therefore eliminate this possibility.

Despite the non-involvement of molecular oxygen in the formation of 5HMdUrd, it is possible that the 5-methyl radical be its radical precursor. This radical is reducing in nature, through the following resonance.

Oxidation of the thymidyl radical has been proposed to lead to the formation of 5HMdUrd (Figure 1.37) (83). Suitable oxidising species present in the sample are various secondary radicals, in particular 5-yl radicals.
Specific formation of the 6-hydroxy-5,6-dihydrothymid-5-yl radical by gamma irradiation of thymidine bromohydrin also yields this product in significant quantities. Here also it was demonstrated that molecular oxygen is not involved. The mechanism may not however be the same in this case since a bromohydrin radical cation has been shown to be formed which may be responsible for its formation (vide infra).

1.6.2. The thymidine radical anion

The electron liberated by photoionisation of a thymidine molecule may be captured by a neighbouring nucleoside. The added electron will reside in a π-orbital, notably the lowest unoccupied molecular orbital (LUMO) and being consistent with the previous nomenclature is termed a π-anion regardless of the original charge state of the molecule. The unpaired electron resides primarily at positions C6, C4 and C2, C6 having the highest unpaired electron density. The principal event leading to restitution of the initial charge state of the molecule is protonation. Although good evidence has been obtained demonstrating protonation of the radical anion at heteroatoms in various glasses, processes which may well occur in the present system, proton additions to C6 are those leading to chemical modification and are therefore of primary interest.

1.6.2.1. 5,6-dihydrothymidine

Protonation of the thymidine radical π-anion at C6 yields the 5,6-dihydrothymid-5-yl radical whose free base analogue was characterised as early as 1963 (84) and is probably the most extensively
documented nucleic acid radical. The above authors prepared the radical by hydrogen atom addition to C6 of an excited thymine molecule, and it was Ormerod in 1965 (85) who first suggested protonation of the thymine radical anion as the likely process involved in the gamma irradiation of DNA. The 5,6-dihydrothymid-5-yl radical formed may subsequently gain a hydrogen atom to yield the quantitatively important 5,6-dihydrothymididine probably by electron transfer and protonation as in the case of the "hydrates". In 1968, Holroyd and Glass (86) demonstrated that in sodium hydroxide glasses the thymine π-anion protonates at C6 and that the proton gained originates from water, and we have demonstrated that this is also the case under the conditions of the presents study. We may note also non-stereospecific addition of the hydrogen atom at C5, the 5R and 5S diastereoisomers being formed in approximately equal amounts.

Catalytic hydrogenation of thymidine using a rhodium/alumina catalyst yields exclusively the 5S isomer, however the use of palladium on charcoal support yields a mixture of both. It is interesting that it has recently been found that formation of 5,6-dihydrothymididine in gamma irradiated DNA yields preferentially the 5R diastereomer (87).

1.6.2.2. 5,6-dihydrothymine

The precursor for the formation of 5,6-dihydrothymine is again likely to be the π-anion undergoing subsequent protonation at C6. It should be noted that the fairly important yield of this product cannot be explained by the secondary radiolysis of thymine released during the irradiation of thymidine. We must therefore explain the loss of the osidic moiety during saturation of the 5,6-double bond. It has been shown that base centred radicals may induce strand breaks and base loss in polynucleotides (79) and has been suggested to occur in DNA (80). In these cases, hydrogen abstraction does not occur within the sugar attached to the base radical in question but from that on the adjacent nucleotide unit. We have demonstrated that the hydrogen atom added to C5 is an exchangeable one (i.e. from hydration water or NH and OH functions on the substrate) by observation of the incorporation of a deuterium atom at both C5 and C6 when thymidine was irradiated in frozen D₂O solution.

We might consider simple hydrolysis of 5,6-dihydrothymididine during separation since saturation of the base is known to weaken the
N-glycosidic bond. The results would appear to rule out this hypothesis. Firstly, 5,6-dihydrothymidine appears to be highly stable in aqueous solution at neutral pH. Secondly, a quantitative study (vide infra) where \(^{14}\text{C}\) labelled thymidine was chromatographed immediately after annealing demonstrated substantial formation of 5,6-dihydrothymine as a direct result of the radiolysis.

We might finally consider a secondary radiolytic process which may be related to a difference in the radiosensitivities of thymidine and 5,6-dihydrothymidine. If the latter, for the same dose of radiation, undergoes a considerable increase in the extent of N-glycosidic link rupture compared to thymidine, we may detect a significant yield of 5,6-dihydrothymine. This difference in radiosensitivity would necessarily be of the order of two orders of magnitude for such a high relative yield of the saturated base to be observed. In support of this hypothesis is the fact that the N-glycosidic link of saturated pyrimidines is weaker than that of the unsaturated nucleoside.

1.6.2.3. 5-Hydroxy-5,6-dihydrothymidine

The formation of 5-hydroxy-5,6-dihydrothymidine is also accounted for by the initial protonation of the thymidine radical anion at C6. This time however the 5-yl radical does not gain a hydrogen atom \((\text{e}^- + \text{H}^+)\). Instead, as in the case of the thymidine diols, we might suggest addition of molecular oxygen to yield the corresponding peroxy radical which is subsequently reduced and undergoes homolytic degradation to give the alcohol.

In a recent paper (74), the 5,6-dihydrothymin-5-yl radical was formed selectively by gamma irradiation of 5-bromo-5,6-dihydrothymine. In the presence of oxygen, ESR studies showed that this radical is converted at probably diffusion controlled rates to the peroxy radical by oxygen addition at C5. The authors also pointed out that this reaction is largely quenched by removal of oxygen but that degassing is never 100 % efficient illustrating the difficulties involved in deoxygenation procedures. The proposal of this mechanism in the formation of 5-hydroxy-5,6-dihydrothymidine was tested by irradiating thymidine in the presence of oxygen enriched in \(^{18}\text{O}\) (vide supra) and indeed incorporation of the heavy isotope was observed although only at
approximately 30%, and as for the thymidine diols, both an indirect effect and a direct effect mechanism involving molecular oxygen appear to contribute to the observed yield of this product. As expected, both the 5R and 5S diastereoisomers were isolated indicating non-stereospecific addition of oxygen or the hydroxyl radical.

1.6.2.4. A contribution by the indirect effects

It would seem appropriate to make certain comments concerning the mechanisms of the formation of some of the products discussed so far. Those mentioned involving saturation of the base moiety may be accounted for, as shown, by typically direct effect processes, but it is also important to keep in mind that these are also products of the indirect effects. It may, we feel, be assumed that the water radiolysis products produced in the bulk solution are effectively kept to the ice crystallites. However, studies have shown (48)(49) that there exist significant quantities of fairly mobile hydration water which may in some cases contribute to the observed yields of the above products.

The only indication we have as to the importance of this contribution has been obtained from the irradiation of thymidine in the presence of $^{18}$O$_2$. For both the thymidine diols and 5-hydroxy-5,6-dihydrothymidine, the incorporation of $^{18}$O at C5 was 30% as measured by mass spectrometry. Care was taken to degass the solution completely under vacuum before saturation with $^{18}$O$_2$ of 99% isotopic purity. Allowing a generous 5-10% $^{16}$O$_2$ content of the solution before freezing and irradiation due to insufficient degassing or leakage of air into the flask during saturation, we can only account for approximately 50% of the observed yield of these products via a direct effect mechanism involving oxygen. Part of the remaining yield of these products may arise from competitive oxidation-reduction processes between the primary and secondary radicals formed. Although we cannot rule out a possible contribution from the indirect effects due the presence of mobile hydration water molecules, we should note that the contribution of the indirect effects to the yield of 5,6-dihydrothymidine is probably insignificant compared to the direct effect mechanism. ESR studies have shown that the radical observed on irradiating thymidine in frozen aqueous solution is the 5,6-dihydrothymid-5-yl radical uniquely, and the indirect effects involve hydrogen addition to both C5 and C6 of
thymidine in similar amounts. In subsequent discussions, the direct effects will be assumed to be the major process.

1.6.4. Oxidic radicals

1.6.4.1. 5',6-cyclo-5,6-dihydrothymidine

The likely mechanism leading to the formation of the 5',6-cyclo-5,6-dihydrothymidines is that shown in Figure 1.38. The initial event involves the loss of a hydrogen at C5'. The 5'-yl radical then undergoes intramolecular attack at C6 to yield the 5',6-cyclo-5,6-dihydrothymidine 5'-yl radical which gains a hydrogen atom in the usual way by electron transfer and protonation via hydration water. Support for this hypothesis came from the incorporation of a deuterium atom at C5 when thymidine was irradiated in frozen deuterium oxide solution. The NMR analysis was made too rapidly for the observed deuterium at C5 to have arisen from slow exchange.

![Figure 1.38: Probable mechanism for the formation of 5',6-cyclo-5,6-dihydrothymidine.](image)

Six stereoisomers of this product have been isolated of the possible eight (three asymmetric carbons).

5',6-Cyclopyrimidines have been documented but all thymidine products still contain the 5,6-double bond. Otter et al (69) reported
the formation of 5',6-cyclo-5,6-dihydouridine as a by-product of the synthesis of 5',6-cyclouridine.

1.6.4.2. Thymidine 5'-aldehyde

The formation of a radical centred at the C5' position is also supported by the isolation of the 5'-aldehyde (Figure 1.39). In addition to the mechanism mentioned in the figure, we might also envisage addition of molecular oxygen to the 5'-yl radical with subsequent elimination of the superoxide radical HO^-. This product was also shown recently to be formed in TpA irradiated by a high energy electron beam in the solid state. Its structure was confirmed by synthesis of the authentic product by mild oxidation of the 3'-acetylated nucleoside with N,N-dicyclohexylcarbodiimide in DMSO.
1.6.4.3. 1-(2-deoxy-\(\alpha\)-L-threo-pentafuranosyl)thymine

The \(\alpha\)-lyxo form of thymidine arises from epimerisation of thymidine at the C4' position. Its isolation would appear to indicate the formation of a C4' centred radical via hydrogen abstraction. Epimerisation of this radical followed by reduction by an appropriate hydrogen donor (Figure 1.40) would yield the final product. This product was synthesised by an enhanced keto-enol tautomerism of thymidine 5'-aldehyde to give a mixture of the normal \(\beta\)-D-erythro and the epimerised \(\alpha\)-L-threo forms.

Simple reduction of the aldehydes with sodium borohydride yields thymidine and its \(\alpha\)-lyxo form which were easily separated by reverse phase HPLC. The product isolated from the gamma irradiation mixture gave an identical \(^1\)H NMR spectrum to the synthesised sample. It is noteworthy
that the pyranoid forms of thymidine which are also associated with a C4' centred radical were not observed in the present study.

It would seem unlikely that such an epimerisation may occur in gamma irradiated DNA owing to the presence of the phosphoester linkage to the continuing DNA strand which would tend to restrict epimerisation. However, the formation of a C4' centred radical may well lead to strand breaks by a $\beta$ elimination mechanism (88) (Figure 1.41).

Figure 1.41: Mechanism of strand break formation via hydrogen abstraction at C4'.

1.6.4.4. Thymine

The important release of thymine suggests generation of sugar centred radicals in significant quantities. Radicals centred at C1', C3' and C4' may all lead to scission of the N-glycosidic bond. Apurinic and apyrimidinic (AP) sites are among the most common base lesions. Such sites occur by spontaneous hydrolysis of the N-glycosidic bond, the rates for which under physiological conditions are $10^{-6}$ for purines and $5\times10^{-8}$ for pyrimidines (89). The twenty-fold difference in the hydrolysis rates of purines and pyrimidines reflects the higher lability of the N-glycosidic bond for the former. It has been calculated from the rates of depurination measured in vitro with purified DNA that a mammalian cell containing $2\times10^9$ nucleotides spontaneously loses 10000 bases from
its genome in 24 hours (90). In addition to this spontaneous base loss, base alkylation (91), as well as UV and ionising radiations lead to increased base loss rates. Also, AP sites may be induced enzymatically by the excision of damaged bases by specific glycosylases (92). Although base loss is an important DNA lesion, the cell appears well equipped to deal with them having at least two repair mechanisms at its disposal. The first involves the well-known excision repair mechanism involving an endonuclease specific for the missing base producing a single strand break in the vicinity of the AP site, with subsequent excision, polymerisation and ligation steps to restore the DNA integrity (93) (94). The second involves a base insertion repair where apurinic sites have been reported to be filled directly using the appropriate deoxyribonucleoside triphosphate by enzymes named purine : apurinate-DNA-purine transferase or simply purine insertase (95).

1.6.4.5. 2-deoxy-D-ribo-1,4-lactone

This product implies more specifically the formation of a radical centred at the Cl' position. The mechanism generally assumed to account for its formation in aerated samples involves addition of molecular oxygen to the Cl' radical with subsequent degradation of the peroxide radical and concomitant release of the base moiety. This mechanism would appear not to be the one which applies in this case, since when thymidine was irradiated in the presence of $^{18}\text{O}_2$, no heavy isotope was shown to be incorporated by electron impact mass spectrometry. We must perhaps look towards a hydration water molecule as the source of oxygen addition at the Cl' position via oxidation of the Cl' radical to form the carbocation with subsequent reaction with water (Figure 1.42).
1.6.5. Radical combination processes

This section describes the formation of various dimeric products resulting from combination of monomeric radicals formed by hydrogen abstraction in the sugar moiety or by deprotonation or protonation of radical π-cations or π-anions respectively. The value of the discovery of such compounds lies in the strong evidence they provide for the presence of the radicals proposed to be involved in the mechanisms of their formation. In particular, the demonstration of the formation of an N3-centred radical provides good proof of the deprotonation of the thymidine radical π-cation at N3, a process for which until now no direct evidence had been obtained. Although the yields obtained are very low compared to monomeric products, it should be reminded that dimeric products, and notably the Pyr(6-4)Pyo adducts, have been suggested to be mutagenic hotspots.

1.6.5.1. 6-Thymidyl-5,6-dihydrothymidine-dThd(α-6)hdThd

By far the most important radiation-induced biadduct is a structural isomer of the spore photoproducts, this time the thymidyl residue
being attached to C6 of the 5,6-dihydrothymidyl residue and shall be referred to as dThd($\alpha$-6)hdThd. The determination of the mechanism involved in its formation posed a challenging problem.

Initially we envisaged two possible mechanisms as outlined in Figure 1.43. We shall begin our discussion by describing the conditions under which this compound is formed. The two isomers of this product were first isolated and identified from gamma irradiated frozen solutions of thymidine and shown to be the major adducts, the spore photoproducts being quantitatively less important. We subsequently searched for these compounds in UV irradiated frozen solutions of thymidine without success. This product was finally shown to be a thymidine photoproduct by irradiation as a thin film with 254 nm UV light. Under these conditions the major adducts are the two isomers of the spore photoproduct, the dThd($\alpha$-6)hdThd products being formed in a yield approximately two orders of magnitude smaller than the spore photoproducts. We may explain the failure to observe the dThd($\alpha$-6)hdThd adducts when thymidine is irradiated with 254 nm UV radiation in frozen aqueous solution in two ways. Firstly, it would not be unreasonable to
suggest that the processes occurring under frozen solution and thin film conditions may be different. Alternatively, if the relative yields of the two types of adduct are the same under both irradiation conditions, since the spore photoproducts are quantitatively minor products in frozen solution compared to the cyclobutyl dimers and dThd(6-4)dPyo adducts, the yields of dThd(α-6)hdThd would not be detectable.

The fact that these products are formed under exposure to both ionising and exciting radiations meant that we could not rule out either a concerted or a consecutive mechanism with any confidence.

Since under the effects of ionising radiation we expect consecutive radical mechanisms to predominate, we tested this hypothesis first. In addition, Bernhard (76) explained the quintet ESR signal observed by Gregoli et al (47) after gamma irradiation of 1-methylthymine crystals by the structure given below, analogous to the nucleoside radical adduct implicated in the consecutive formation of dThd(α-6)hdThd.

Thymidine was irradiated with $^{60}$Co gamma rays under the usual conditions of temperature and concentration but in $D_2O$ solution (vide supra). If the mechanism involves attack by a methylene radical at C6 of an adjacent nucleoside molecule to yield the 6-thymidyl-5,6-dihydrothymid-5-yl radical, we expected the latter to abstract a deuteron from a hydration water molecule in a manner similar to the 5,6-dihydrothymid-5-yl radical in the formation of 5,6-dihydrothymidine. Both isomers of
dThd(α-6)hdThd were shown by 400 MHz $^1$H NMR spectroscopy to have a proton at C5 suggesting that the 5-yl radical is not an intermediate in their formation.

The second possibility involves a concerted mechanism. We have also shown that when thymidine is exposed as a thin film to far UV light, the spore photoproduct is the major adduct and that two mechanisms are apparent, one of which is concerted (65%) and the other consecutive (35%). This was demonstrated by the synthesis and subsequent UV irradiation of thymidine completely deuterated at the methyl group (vide infra). We showed by $^1$H NMR spectroscopy that the spore photoproduct obtained contained a deuterium atom at C6 of the saturated residue at 65% with complete stereospecificity demonstrating proton transfer from the methyl group of one residue to the C6 position of the other. We decided to use this approach to determine whether the same type of process takes place during the formation of the dThd(α-6)hdThd adducts under the direct effects of gamma radiation. The results reported in Section 1.5.2. showed conclusively that the mechanism is non-concerted.

A possibility which we might consider is that the 5-yl radical abstracts a non-exchangeable proton from a neighbouring nucleoside. We find it difficult however to reconcile this with the observed behaviour of the 5,6-dihydrothymid-5-yl radical which was shown to gain an electron followed by protonation from water or an exchangeable proton site in a neighbouring substrate molecule. A more likely process, and that which we tend to favour, would involve the addition of a hydrogen atom at C5 of a thymidine molecule to form the 5,6-dihydrothymid-6-yl radical which may subsequently undergo radical combination with a 5-(2'-deoxyuridilyl) methyl radical to give the stable diamagnetic product (Figure 1.44).
Hydrogen atoms have been shown to add prefentially to C5 of thymidine when photolysed in aqueous solution (96). Quantitatively it would appear that hydrogen atom loss from the osidic moiety, especially at the C5' position, is an important process and may be the source of such a hydrogen atom transfer. Studies in prospect include the irradiation of thymidine deuterated at the C5' and C4' position to see whether deuterium transfer from these positions to C5 of thymidine does indeed occur.

Finally we may explain the inversion in the relative yields of the dThd(α-5)hdThd and dThd(α-6)dhThd adducts depending upon the radiation type by the different relative importances of radical and excitation processes.

Structurally, the presence of two asymmetric carbons at C5 and C6 would be expected to yield four diastereoisomers. However only two
isomers have been isolated. In addition, keto-enol tautomerism would be expected to occur at C5 owing to the \( \alpha \)-carbonyl group and indeed this is shown to be the case by a slow exchange (2-3 days) of the C5 proton in D\(_2\)O solution. This tautomerism occurs with retention of configuration at C5 which suggests a preferred configuration due to steric effects between the methyl group and the thymidyl residue at C6. We would expect a \textit{trans} relative configuration between these two bulky groups and indeed this was shown to be the case by the detection of a Nuclear Overhauser Effect between the (\textit{cis}) methyl group and the C6 proton. Hence this configuration would rapidly be assumed in aqueous solution regardless of any stereospecificity in the formation of these products, the final configuration at C5 being governed by that at C6.

1.6.5.2. \textit{dThd}(\(\alpha-\alpha\))\textit{dThd}

The only reasonable mechanism we may propose for the formation of \textit{dThd}(\(\alpha-\alpha\))\textit{dThd} is the simple combination of two radicals formed by deprotonation of the thymidine radical cation at the methyl group (Figure 1.45).

![Figure 1.45](image)

\textit{Figure 1.45} : Probable mechanism for the formation of \textit{dThd}(\(\alpha-\alpha\))\textit{dThd}.

The fact that this product be formed in such significant quantities compared to certain monomeric compounds shows that deprotonation of the
thymidine radical cation at the methyl group is a quantitatively important process, since it is statistically improbable that two such radicals form on adjacent thymidine molecules. This said however, it is by no means necessary that such events occur simultaneously. A remarkable property of these radicals is their stability. Crystals of T.H₂O give CH₂ radicals when irradiated at room temperature, and in gamma irradiated 1-methylthymine they remain even up to 200°C (97). It is possible then that an initially formed radical may wait for the formation and deprotonation of an adjacent radical cation before undergoing radical combination. Considering the high local concentrations produced on phase separation during freezing and the high intensity of the gamma rays used, this is not unlikely.

1.6.5.3. dThd(5'-6)hdThd

Two of the eight possible isomers of this product have been isolated. As for the 5',6-cyclo-5,6-dihydrothymidines, the initial event might involve formation of a C5' centred radical which this time undergoes intermolecular attack at C6 of a neighbouring thymidine molecule. Again the 5-yl radical formed is likely to be reduced to give the carbanion with subsequent proton transfer from a hydration water molecule.

Alternatively, a process similar to that proposed for the formation of the dThd(α-6)hdThd adduct may occur, that is hydrogen atom addition to C5 of a thymidine molecule with subsequent combination with a neighbouring 5'-yl radical.

1.6.5.4. dThd(3-5)hdThd

This compound represents the first clear demonstration of the formation of a radical centred at N3, and has the added interest of providing the first concrete evidence of a deprotonation of the thymidine pristine π-cation at this position. No ESR studies have to date yielded data suggesting this possibility although intuitively one might expect it. The ESR signal obtained for this radical may be of such low intensity that it might be lost under the superimposed signals of other quantitatively more important radicals.
The mechanism is likely to involve the combination of the resulting N3 centred radical with a 5,6-dihydrothymid-5-yl radical formed by protonation of a thymidine radical $\pi$-anion at C6. An alternative would be attack at C5 of the 5,6-double bond of an adjacent thymidine molecule to form a 5,6-dihydrothymid-6-yl radical which would subsequently be reduced. If this type of reaction were to be that involved, since C6 is just as favourable for such an attack, if not more so due to the steric hindrance between the C5 methyl group and the incoming bulky radical, we would expect to observe a similar if not greater yield of the dThd(3-6) hdThd isomer. This product was not isolated. Not only does this finding support the proposed mechanism, it also would appear to indicate that the 5,6-dihydrothymid-6-yl radical formed by an indirect effect involving mobile hydration water is quantitatively far less important than the corresponding 5-yl radical and we may assume that this product and the 5,6-dihydrothymidines are primarily the result of direct effect processes. Also, the 6-yl radical which has been proposed to be involved in the formation of dThd(\(\alpha\)-6)hdThd is not formed via an indirect effect mechanism since in frozen D$_2$O, the product did not show the expected incorporation of deuterium at C5.

The probability of the occurrence of two such radicals on adjacent molecules, i.e. N3 and C5 centred radicals, may be enhanced by the nature of the initial charged species. It is amusing to consider the two original nucleosides in a closed system. It is known that the electron displaced by the incident gamma photon does not travel very far before being recaptured by the radical cation formed or by a neighbouring nucleoside. We may envisage then the formation of a radical anion-cation pair. We may also imagine that the deprotonation of the $\pi$-cation at N3 facilitate the protonation of the $\pi$-anion at C6 either by direct transfer of the N3 proton or more likely through the intermediary of hydration water molecules. Transfer may of course occur in the opposite direction with the same result, i.e. abstraction of a hydration water proton which results in a transfer of an "aprotonic" hole eventually inducing proton loss at N3 of the $\pi$-cation.

As might be expected, both the 5R and 5S isomers have been isolated indicating non-stereospecific radical combination.
1.6.5.5. dThd(α-3)dThd

The assignment of this structure is still tentative. We may however envisage the formation of such a molecule by the combination of a 5-(2′-deoxyuridilyl)methyl radical with an N3 centred radical formed by the deprotonation of a thymidine radical cation.

1.6.6. Excitation processes

1.6.6.1. The cyclobutane dimers

The cyclobutane dimers are typical photoproducts of the pyrimidines resulting from the 2+2 addition of the 5,6-double bonds. We would therefore naturally look to excitation processes to explain their formation in the present case. The gamma irradiation of thymine in frozen aqueous solution has been shown to yield the cis/syn diastereoisomer of the thymine cyclobutyl dimer (98). The literature also contains numerous articles on the induction of photoreactivatable (PR) damage in cells exposed to ionising radiation. The situation has recently been reviewed (99).

The formation of PR damage, which is unique to pyrimidine dimers, was initially assigned to a Cerenkov emission. A radical mechanism was ruled out on the basis of an apparent lack of effect of free radical reactive agents such as oxygen (100) and thiothreitol (101) on the yield of PR damage. It was also observed by several groups that the yield of PR damage was dependent on the energy of the photon beam and below a certain value little or no such damage was produced. Increasing the energy above 300 kVp resulted in an increasing PR damage.

Cerenkov emission occurs when a particle exceeds the speed of light in the medium through which it travels; in most cases the particle involved is an ejected electron. Theoretical calculations have shown that the cut-off energy for an electron producing a Cerenkov emission in water is 236 KeV. This corresponds to a minimum incident photon energy required to create a recoil electron of such an energy of 300 KeV assuming a 180° back scattering of the photon.
Evidence also exists showing that PR damage may be induced by mechanisms not involving Cerenkov radiation and comes mainly from experiments carried out using radiation of insufficient energy to produce a Cerenkov emission. The mechanism involved in this case is not clear, however direct excitation of the substrate or possibly a UV emission by excited molecules on an electron track have been proposed. Strong evidence for the former has been provided by Moss and Smith (102) when exogenous DNA added to the cell suspension was shown to have no effect on the yield of PR damage of 50 KVp X-rays, the DNA being expected to absorb the damaging component of the Cerenkov emission and any radiation produced by molecules along the electron tracks.

We must now consider the relative contributions of Cerenkov emission and direct excitation in the irradiation of thymidine in frozen aqueous solution with $^{60}$Co gamma rays. The gamma rays used are certainly of sufficient energy to produce a Cerenkov emission. It has been shown that the induction of PR damage increases with increasing pathlength of the photon beam before reaching the substrate. In the present study, the sample was irradiated in a stainless steel capsule at the bottom of a 3 m pool. The capsule would absorb all external Cerenkov radiation and the remaining volume available for Cerenkov emission would be the sample volume of about 150 cm$^3$. From a quantitative point of view, the study described in Chapter IV on the effects of 254 nm light on thymidine irradiated under identical conditions of temperature and concentration yields valuable information. The fluences of UV radiation used in this experiment were far greater, one would estimate by a few orders of magnitude, than any Cerenkov emission source and yet we note at most one order of magnitude difference in the yield of dimers. Although a Cerenkov emission almost certainly contributes to the observed yields of the cyclobutyl dimers, we feel that by far the major contribution arises from direct excitation of the substrate.

1.6.6.2. The spore photoproducts

Having isolated the cyclobutane dimers, and having associated their formation with excitation processes, it is not surprising that we have also isolated the 5R and 5S diastereomers of the spore photoproduct. 5-Thymidyl-5,6-dihydrothymidine or dThd($\alpha$-5)dThd was first shown to be
formed in UV irradiated frozen aqueous solutions of thymidine by Varghese (103) and confirmed in the study described in Chapter IV. Empirically, the relative yields of the cyclobutane dimers and the spore photoproducts in the gamma irradiation experiments are similar to those obtained in the UV studies based on HPLC peak intensities and we may therefore link their formation to the same excitation processes. It is true that we might also reasonably propose the combination of a 5-methyl radical with a 5,6-dihydrothymid-5-yl radical for whose formation we have clear evidence. As discussed later, 254 nm UV irradiation of thymidine in frozen D$_2$O solution does not result in deuterium incorporation at C6 thereby ruling out addition of an exchangeable proton at this position. The confirmation of the occurrence of a concerted process in the gamma induced formation of the spore photoproduct was obtained by gamma irradiating thymidine fully deuterated at the methyl group. $^1$H NMR spectrometry demonstrated deuterium incorporation at C6 in this case indicating transfer of a methyl hydrogen (Figure 1.46).

![Figure 1.46](image)

**Figure 1.46**: Concerted mechanism for the formation of the spore photoproduct.

The evidence obtained overwhelmingly points to the involvement of excitation processes only during the formation of the spore photoproduct in gamma irradiated frozen aqueous solutions of thymidine similar to those occurring when thymidine is irradiated under identical conditions with 254 nm UV light.
CHAPTER II

THE DIRECT EFFECTS OF GAMMA RADIATION ON 2'-DEOXYCYTIDINE

2.1. Introduction

Continuing the study of the direct effects of gamma radiation on 2'-deoxyribonucleosides, we transferred our attention to the other pyrimidine nucleoside, 2'-deoxycytidine. Although the radiation chemistry has been fairly extensively studied using the ESR technique with crystalline or glassy samples at low temperatures, studies of the stable degradation products resulting from the indirect effects, that is the reaction of water radiolysis products, are few, and those carried out on the direct effects are, to our knowledge, non-existent.

The major proportion of current knowledge of the indirect effects have been obtained by Polverelli and co-workers (104-109) on the degradation of the free base, cytosine, in the presence of oxygen. Decarroz and Cadet (110) have recently extended the study to the 2'-deoxyribonucleoside, and of particular interest and relevance to the present work on the direct effects, by the menadione photosensitised oxidation of 2'-deoxycytidine producing the corresponding radical cation. The results of this work allow an interesting comparison with the products linked to the formation of the radical cation during the gamma irradiation of frozen solutions exposed in this chapter.

In the present work, 2'-deoxycytidine has been irradiated in two forms, as the free base nucleoside and as its hydrochloride salt. The latter study was carried out first for reasons of increased solubility in water and hence shorter irradiation times. The discovery of certain products which may result from the acid induced rearrangement of the main radiolysis product 5,6-dihydro-2'-deoxyuridine, required us to irradiate 2'-deoxycytidine. As well as resolving the question of the involvement of low pH in the formation of these products, the study of the direct effects of gamma radiation on both substrates provides additional information on the importance of the initial charge state of the molecule in the subsequent degradation of the $\pi$-anions and $\pi$-cations formed.
2.2. ESR studies on the radiation induced radical species of cytosine and its derivatives

As for thymidine, exposure to ionising radiation in the solid state leads to the formation of radical cations and anions. The modes of degradation of these species however differ appreciably.

2.2.1. The cytosine \( \pi \)-cation

Evidence for the formation of cytosine \( \pi \)-cations has been obtained in a number of laboratories, the clearest of which was obtained by Sevilla et al (111) for the photoionisation of 5-methylcytosine in frozen glasses. As for thymine, the preferred site of deprotonation is at the N1 position. However, for an N1 substituted cytosine, such as the nucleoside, deprotonation occurs at the exocyclic amino group at C4, and at the Cl' position. In the case of 5-methylcytosine which is also substituted at the N1 position, deprotonation at the methyl group occurs in a manner analogous to the thymidine radical \( \pi \)-cation. The deprotonation pathways of cytosine \( \pi \)-cations are resumed in Figure 2.1. Westhof et al (112) showed that hydroxyl anion adds to C6 of the \( \pi \)-cation formed during irradiation of C.H\(_2\)O crystals, and also that the proton liberated adds to the N3 position (Figure 2.2).

2.2.2. The cytosine \( \pi \)-anion

Unlike thymine \( \pi \)-anions, the principal sites of protonation are at heteroatoms rather than the 5,6-double bond. Good evidence exists for the addition of a proton at the N3 (113) and O2 (114) positions. Addition to C6 appears to occur with protonation at N3 also. The preference for protonation at N3 or O2 depends on the proton donor environment. In the C.H\(_2\)O crystal, protonation occurs to O2 rather than to N3 and then C6 (115). This has been accounted for by the fact that in these crystals N3 is bonded to N1 of an adjacent cytosine, a poor hydrogen donor, whereas O2 behaves as a hydrogen bond acceptor to two water protons and one N4 amino proton (116). Evidence for the conversion of the O2-proton adduct to a C5 adduct upon annealing to room temperature has been obtained by Westhof et al (117). The radical events following electron capture are outlined in Figure 2.3.
Figure 2.1: Deprotonation pathways for the cytosine radical cation.

Figure 2.2: Hydration of the cytosine radical cation.
Figure 2.3: Protonation pathways of the cytosine radical anion.
2.3. Isolation of the final degradation products of 2'-deoxycytidine and its hydrochloride salt

The general analytical approach used was essentially that used in the study of the direct effects of gamma radiation on thymidine and described in Section 1.3. The only difference was that this time an initial fractionation on a preparative column was deemed not necessary. The number of products formed was much lower than that found for thymidine, and more importantly, no products were observed which are better retained on the reversed phase column than 2'-deoxyuridine, attributable to the lack of dimeric compounds due to the absence of a methyl group in the parent molecule. In the absence of a rigorous quantitative study on the radiation induced degradation of 2'-deoxycytidine, we are unable to say whether the overall degradation for this pyrimidine nucleoside is greater for a given dose than for thymidine.

The initial step was comprised of successive crystallisations from hot ethanol resulting in a mixture which could be analysed directly by analytical reversed-phase HPLC. The fact that products of 2'-deoxycytidine and 2'-deoxyuridine are generally more weakly retained than the corresponding derivatives of thymidine meant that the most polar eluent, that of water, was necessary for the initial fractionation. The fractions thus obtained were subsequently subjected to silicagel HPLC using the Solvent II eluent.

2.4. Identification of the products resulting from the direct effects of gamma radiation on 2'-deoxycytidine and its hydrochloride salt

The following products are known compounds readily available commercially and their identification as products of the gamma radiolysis of 2'-deoxycytidine was made by simple comparison of their $^1$H NMR spectra with those of the authentic samples.

1. 2'-Deoxyuridine
2. Cytosine
3. 5,6-Dihydrouracil.

2'-Deoxyribonolactone is a well-known product of the OH' mediated degradation of 2'-deoxyribonucleosides and gives a characteristic $^1$H NMR spectrum.
3.4.1. 5,6-dihydro-2'-deoxyuridine

To our knowledge, the α- and β-furanoid and the α- and β-pyranoid forms have never been characterised in the literature. The structural analysis of these products was made by mass spectrometry and more importantly by $^1$H NMR which distinguishes clearly the different configurations of the sugar moiety, and which allows ready comparison with the spectra of other nucleosides having undergone rearrangement of the sugar moiety. The pyranoid anomers of 2'-deoxyadenosine are known products of the indirect effects (1) and of the direct effects (2) of gamma radiation on this purine nucleoside and have been characterised (118). The features of the corresponding products of 5,6-dihydro-2'-deoxyuridine are very similar and are listed in Table II.1 along with the relevant values obtained for the corresponding 2'-deoxyadenosine products.

The $^1$H NMR spectra show the loss of the vinyl proton signals at low field with the introduction of two base ring CH$_2$ groups presenting tight or degenerate ABMN systems. The C5 methylene group presents in each case a pseudotriplet at 200 MHz in the region of 2.75 ppm and the C6 methylene group also gives a pseudotriplet at a chemical shift in the region of 3.6 ppm which compares well with that of 5R and 5S 5,6-dihydrothymidine (61).

The structural analysis is made by close examination of the coupling constants between sugar protons. For the pyranoid isomers, the anomic proton signal generally presents a double doublet showing a large coupling to the trans proton at the C2' position. For the α-anomer this is the H2" proton and for the β-anomer the H2' proton. In each case the cis coupling constant is small, in the region of 2 Hz. The α-anomer is easily distinguished by the coupling pattern of the H2" signal which shows a strong coupling to both H1' (11.30 Hz) and H3' (11.58 Hz). These values can only be explained for $^1$C$_4$ conformer where the H1', H2" and H3' protons are in trans - axial positions and the base adopts an equatorial position. The weak couplings of the H4' proton to the H5' and H5" protons indicates a pseudo-equatorial orientation in the α-anomer. For the β-anomer, we note a strong trans - diaxial coupling between H4' and one of the 5' protons (H5") consistent with a $^4$C$_1$ conformation, again the base lying in a preferentially pseudo-equatorial position. In the cases of the furanoid forms, the assignment of a spectrum to a particular anomer is less straightforward since for most of the osidic protons the
### TABLE II.1

1H NMR PARAMETERS FOR THE FURANOID AND PYRANOID FORMS OF 5,6-DIHYDRO-2'-DEOXYURIDINE AND 2'-DEOXYADENOSINE.

<table>
<thead>
<tr>
<th></th>
<th>5,6-DIHYDRO-2'-DEOXYURIDINE</th>
<th>2'-DEOXYADENOSINE (118)</th>
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<tbody>
<tr>
<td></td>
<td>β-FURANOID</td>
<td>α-FURANOID</td>
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<tr>
<td>δ1'</td>
<td>6.31 a</td>
<td>6.16</td>
</tr>
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<td>2.36</td>
<td>2.61</td>
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<tr>
<td>δ2''</td>
<td>2.17</td>
<td>2.05</td>
</tr>
<tr>
<td>δ3'</td>
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<td>4.38</td>
</tr>
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<td>δ5''</td>
<td>3.71</td>
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</tr>
<tr>
<td>δH6</td>
<td>3.58</td>
<td>3.66</td>
</tr>
</tbody>
</table>

- a - chemical shifts in ppm from TSP (2-trimethylsilylpropionate-2,2,3,3-d4) as internal reference
- c - δ2' and δ5' refer to the methylene endo protons for the pyranoid forms.

### TABLE II.2

PRINCIPAL FRAGMENTATIONS OF THE MOLECULAR ION OF 5,6-DIHYDRO-2'-DEOXYURIDINE.

<table>
<thead>
<tr>
<th>FRAGMENT</th>
<th>M/Z</th>
<th>β-FURANOID</th>
<th>α-FURANOID</th>
<th>β-PYRANOID</th>
<th>α-PYRANOID</th>
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<td>M-H2O</td>
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<td>1.0</td>
<td>2.0</td>
<td>3.8</td>
<td>3.6</td>
</tr>
<tr>
<td>M-2H2O</td>
<td>194</td>
<td>87.7</td>
<td>100.0</td>
<td>7.6</td>
<td>3.9</td>
</tr>
<tr>
<td>M-CH2OH</td>
<td>199</td>
<td>21.7</td>
<td>20.6</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>BH-CH=CH2</td>
<td>142</td>
<td>94.0</td>
<td>51.5</td>
<td>25.7</td>
<td>23.6</td>
</tr>
</tbody>
</table>
multiplicities and coupling constants are fairly typical of 2'-endo β-furanoid 2'-deoxyribonucleosides. The β-furanoid anomer is assigned by comparison of the chemical shifts and coupling patterns observed with those of the authentic β-furanoid 5R and 5S 5,6-dihydrothymidine (61). The coupling patterns and especially the chemical shifts of the osidic protons are effectively identical for each since as expected the presence of the methyl group at C5 in the case of 5,6-dihydrothymidine does not have an important effect on the conformation of the molecule. On the other hand, it has been shown that the conformation of the molecule is highly sensitive to the nature and orientation of a C6 substituent.

The mass spectra of each isomer support the $^1$H NMR structural analysis. Two methods were used. Since the molecular ions are highly unstable under normal electron impact mass spectrometry (EIMS) conditions, the latter spectra show peaks of very low intensity at m/z 231 corresponding to the protonated parent molecule. The molecular mass was confirmed less unambiguously by DCI-MS (desorption chemical ionisation mass spectrometry) where the sample is placed on a metal filament and heated very rapidly in an atmosphere containing ammonium cations. The spectra obtained for this series of products are effectively identical and the only significant peaks are those having masses of m/z 231 and m/z 248 corresponding to MH$^+$ and MNH$_4^+$ respectively. Figure 2.4 shows the DCI mass spectrum for the β-furanoid form.

The EIMS spectra of the four isomers are shown in Figure 2.5a-d. The trends obtained agree very well with those reported in the literature for the furanoid and pyranoid forms of thymidine and its bromohydrin (120). Table II.2 lists the primary molecular ion fragmentations for each isomer. The loss of one molecule of water is slightly more favorable for the pyranoid isomers, however the concerted loss of two molecules of water from the sugar moiety is enhanced for the furanoid forms, in the case of the α-furanoid isomer this fragmentation leads to the base peak ion. The loss of the 5'-hydroxymethyl group is only significant for the furanoid forms and this process readily distinguishes the two osidic configurations. In addition, this fragmentation is slightly favored for the β-isomer and is linked to the proximity of the base residue to the 5'-hydroxymethyl group in the latter. The rearrangement fragmentation yielding the BH-CH=CH$_2^+$ fragment (m/z 141) as is the cleavage of the N-glycosidic bond (S$^+$ m/z 117, BH$_2^+$ m/z 115, BH$^+$ m/z 114) are also enhanced for the furanoid forms again in good agreement with the literature (120).
Form of 5,6-dihydro-2'-deoxyuridine.

Figure 2.4: The DDI mass spectrum of the 6-Furandiol
Figure 2.5: The FAB mass spectra of the furanoid and pyranoid forms of 5',6-dihydro-2'-deoxyuridine.
2.4.2. 5',6-cyclo-5,6-dihydro-2'-deoxyuridine

All four of the theoretically possible isomers have been isolated during this study and analysed by $^1$H NMR spectrometry (Figure 2.6). The chemical shifts and coupling constants of the four isomers are listed in Table V.3. Each isomer presents characteristics typical of 5',6-cyclopyrimidine and 5',8-cyclopurine nucleosides. We note coupling constants tending towards zero for the H1' H2' and H3' H4' pairs, and long range coupling between H2" and H4'. The base residue is now saturated as indicated by the loss of the downfield H5 and H6 vinyl signals of the parent nucleoside. We detect instead an ABX system, the AB part in the region of 3 ppm and 2.8 ppm corresponding to the geminal protons at C5 of the base, and the X proton (H6) downfield at around 4 ppm. We note also the presence of only one H5' signal, the assignment of which was made by selective decoupling experiments and which shows coupling constants found elsewhere in the H6 multiplet and the H4' signal. The $^1$H NMR data appear to demonstrate quite unambiguously the formation of a covalent bond between C5' of the osidic moiety and C6 of the base. On standing in D$_2$O solution, we note fairly rapid exchange (2 days) of the C5 protons through keto-enol tautomerism.

A detailed $^1$H NMR analysis has allowed the attribution of a particular configuration to each isomer and is discussed in Chapter V.

2.5. The direct effects of gamma radiation on 2'-deoxycytidine and its hydrochloride salt

The observation in the irradiation of dC.HCl of 5,6-dihydro-2'-deoxyuridines having undergone rearrangement of the osidic moiety was unexpected. The question arose whether such sugar modifications are the result of radical processes subsequent to irradiation, or whether during the isolation process, the presence of the weakly acidic hydrochloride (pKa 4) may have induced ring opening of the sugar. The first possibility is unlikely since base radicals are known not to abstract a hydrogen from within the furanose attached to the base in question. The fact also that all four isomers are formed in approximately equal yields argues against the occurrence of intermolecular hydrogen abstraction. For the second, ring opening of this kind was shown to occur when thymidine was warmed with mineral acids, indeed this proved to be a convenient method for the preparation of authentic samples of the $\alpha$-furanoid and the $\alpha$- and $\beta$-pyranoid forms.
Figure 2.6: 400 MHz $^1$H NMR spectrum of isomer IV of 5',6-cyclo-5,6-dihydro-2'-deoxyuridine.
### TABLE II.3

**CHEMICAL SHIFTS (1) FOR 5',6-CYCLO-5,6-DIHYDRO-2'-DEOXYURIDINES (PPM)**

<table>
<thead>
<tr>
<th>ISOMER</th>
<th>δ1'</th>
<th>δ2'</th>
<th>δ2''</th>
<th>δ3'</th>
<th>δ4'</th>
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**COUPLING CONSTANTS (2) FOR 5',6-CYCLO-5,6-DIHYDRO-2'-DEOXYURIDINES (Hz)**

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<th>1''2''</th>
<th>2'2''</th>
<th>2'3'</th>
<th>2''3'</th>
<th>3'4'</th>
<th>4'5'</th>
<th>5'H6</th>
<th>H5H6</th>
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<td>9.4</td>
<td>5.1</td>
<td>11.8</td>
<td>-17.2</td>
</tr>
<tr>
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<td>5.8</td>
<td>-14.4</td>
<td>6.8</td>
<td>1.8</td>
<td>0.0</td>
<td>8.7</td>
<td>4.4</td>
<td>13.6</td>
<td>5.2</td>
<td>-17.2</td>
</tr>
<tr>
<td>III</td>
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<td>5.5</td>
<td>-14.4</td>
<td>6.6</td>
<td>1.9</td>
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<td>4.8</td>
<td>12.8</td>
<td>-17.1</td>
</tr>
<tr>
<td>IV</td>
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<td>2.9</td>
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<td>2.5</td>
<td>2.9</td>
<td>10.7</td>
<td>5.8</td>
<td>-17.3</td>
</tr>
</tbody>
</table>

1 - Chemical shifts in ppm against TSP internal reference.  
2 - Coupling constants in Hz ±0.1 Hz.
An important point to be taken into account is the lability of the N-glycosidic bond. There is current controversy concerning the mechanism of acid induced ring opening, namely the initial site of protonation, i.e. at N1 of the base or at O4 of the furanose. We should note that products of the unsaturated nucleosides having undergone sugar rearrangement do not occur in detectable amounts. Irradiation of 2'-deoxycytidine under identical experimental conditions yielded products identical to those found for the hydrochloride salt, except that in this case, the only 5,6-dihydro-2'-deoxyuridine found had the natural β-furanoid configuration. This proves that the osidic ring rearrangement observed for dC.HCl was acid induced. Whichever mechanism applies in the acid induced ring opening of the furanose, an increased basicity of the N1 nitrogen will facilitate this process. The mechanism involving protonation of the furanose ring oxygen, which is the most often proposed to explain this phenomenon, is outlined below (Figure 2.7). It is

![Figure 2.7: Acid-induced rearrangement of the osidic moiety.](image)

clear that an increased availability of the N1 lone pair through saturation of the 5,6-double bond of the base ring will facilitate formation of the Schiff's base intermediate and therefore ring opening.
2.6. Mechanistic aspects of the radiation induced degradation of 2'-deoxycytidine

The radiation chemistries of 2'-deoxycytidine and its hydrochloride salt are essentially identical, the events leading to rearrangement of the osidic moiety of 5,6-dihydro-2'-deoxyuridine occurring subsequently to radiolytic processes as described above. We shall divide the discussion of the mechanisms involved in the formation of the various products as usual according to the initial radical species.

2.6.1. The 2'-deoxycytidine π-cation

2.6.1.1. 2'-deoxyuridine

The only product which may be directly related to the π-cation is 2'-deoxyuridine. The likely mechanism involves the deprotonation of the radical cation at the exocyclic amino group with subsequent deamination.

We might also account for the formation of 2'-deoxyuridine by the transient hydration of the 5,6-double bond with subsequent deamination and dehydration. However, the fact that this product was obtained in similar yields for the nucleoside and its hydrochloride salt, the presence of acid being expected to catalyse dehydration, and the knowledge that 6-hydroxy-5,6-dihydro-2'-deoxyuridine is fairly stable at neutral pH, suggest that such a contribution would be minor.

2.6.2. The 2'-deoxycytidine π-anion

As for thymidine, the main reaction of the π-anion is saturation of the base via an initial protonation at C6, although addition at C5 has not been ruled out.

2.6.2.1. 5,6-dihydro-2'-deoxyuridine

Reduction of the 5-yl radical formed by protonation of the π-anion at C6 may occur, as in the case of thymidine, via direct hydrogen addition or via electron transfer to yield the carbanion followed by protonation via hydration water. If the initial protonation of the π-anion occurs at C5, then the
mechanism would involve direct hydrogen transfer only since the corresponding 6-yl radical is reducing, not oxidising as in the case of the 5-yl radical, unless there exists a radical in the vicinity with a higher oxidising potential than the 6-yl radical.

2.6.2.2. 5,6-dihydouracil

Again we may compare the formation of this product with the analogous product of thymidine. It is likely that the initial step involves protonation of the nucleoside π-anion. The proton added to C5 undoubtedly originates from a hydration water molecule, however we cannot say at which stage the N-glycosidic linkage is broken. It is possible, as in the case of the formation of 5,6-dihydrothymine, that the release of this product is a secondary radiation process owing to a possible greater radiosensitivity than the unsaturated starting material.

2.6.3. Osidic radicals

Not unexpectedly, similar osidic radical processes appear to occur for 2'-deoxycytidine as for thymidine under the direct effects.

2.6.3.1. 5',6-cyclo-5,6-dihydro-2'-deoxyuridine

It is clear from the quantitatively important formation of the 5',6-cyclo-5,6-dihydro-2'-deoxyuridines that radical formation at the C5' position is also an important process for 2'-deoxycytidine (cf. Section 1.6.4.1.). Bernhard et al (121) provided evidence for the formation of a 5'-yl radical via hydrogen abstraction at this position when they irradiated 3'-cytidylic acid (3'CMP). Compared to the thymidine analogues, the situation is somewhat simplified since we now have only two asymmetric carbon atoms giving four possible stereoisomers.

2.6.3.2. Cytosine and 2-deoxyribonolactone

Base release is again quantitatively perhaps the most important process indicating the formation of sugar centred radicals. 2-Deoxyribonolactone implies again more specifically the formation of a radical centred at C1'.
This may be the result of hydrogen atom abstraction or by deprotonation of the nucleoside radical cation at this position.

2.7. A comparison of the reaction of the 2'-deoxycytidine radical cation in aqueous solution and in the frozen state.

The radical cation formed by electron transfer to menadione in its excited triplet state in solution at room temperatures undergoes the reactions expected, that is addition of hydroxide ion (water) to C6 and deprotonation (110). Addition occurs to C6, with fast reaction of the resulting 5-yl radical with molecular oxygen yielding the corresponding 6-hydroxy-5-hydroperoxyl radical. Reduction and subsequent dismutation yield the diols, alternatively, β-cleavage of the oxyl radical may give the nucleoside derivatives of carboxamide-α-imidazolidone and biurea. Hydration of the radical cation would not appear to be quantitatively an important process in gamma irradiations in the frozen state since in these experiments no trace of the diols or of the hydrates was observed.

The observation in the photosensitisation experiment of important quantities of 2'-deoxyuridine and of cytosine and 2-deoxy-D-ribo-1,4-lactone fits in nicely with the results of the gamma irradiation of 2'-deoxycytidine in frozen aqueous solution. This suggests strongly that deprotonation of the radical cation occurs at the exocyclic amino group and at the C1' position of the sugar under both sets of experimental conditions.

2.8. A comparison between the direct effects of gamma radiation on thymidine and 2'-deoxycytidine.

Although from the list of the products resulting from the direct effects of gamma radiation on 2'-deoxycytidine, the events involved in its degradation appear less complicated than those for thymidine, there are in fact distinct similarities.

We have not detected the presence of the cyclobutyl dimers of 2'-deoxycytidine or of 2'-deoxyuridine although these are likely to be formed. They are certainly minor products compared to the monomeric compounds and therefore may well be hidden beneath peaks of the latter products during the analytical
reversed-phase HPLC fractionation. Reinjection of a fraction containing these products onto a silicagel HPLC column certainly results in their separation, but being highly retained on this phase using the solvent II eluent, and in view of their low relative yield, the low intensity, diffuse peaks are probably lost in a generally unstable base line. It is also likely that these products be quantitatively less important than the cyclobutane dimers formed in the gamma radiolysis of thymidine since the energy of the triplet state intermediate of cytosine is higher than that of thymidine.

The saturation of the 5,6-double bond to give the corresponding 5,6-dihydronucleoside appears to be quantitatively the most important base modification process for the pyrimidine nucleosides.

Certain products detected for thymidine may also be formed as the analogous 2'-deoxyuridine products. For example, a link between C5' and C6 of two nucleoside units might be envisaged to give dCyd(5'-6)hdUrd since the C5' centred radical appears to be formed in large quantities, as in the case of thymidine radiolysis, as demonstrated by the observation of important yields of the 5',6-cyclo-5,6-dihydronucleoside.

In conclusion, we may make the general comment that the principal radiolytic processes that occur during the gamma radiolysis of 2'-deoxycytidine in frozen aqueous solution are similar to those implicated in the radiolysis of thymidine under the same experimental conditions. We may also note that the final products resulting from the direct effects do not appear to depend on the initial charge state of the base residue since these are the same for the free nucleoside and its hydrochloride salt. We cannot say whether the individual mechanistic steps are all identical.
CHAPTER III

THE DIRECT EFFECTS OF GAMMA RADIATION ON 2'-DEOXYADENOSINE

3.1. Introduction

The far greater part of the research on the indirect effects of ionising radiations on nucleic acid components has focussed on the pyrimidines. This imbalance is beginning to be rectified, however the studies of the final degradation products of the purines, especially of guanine derivatives which are extremely insoluble in water, present certain experimental difficulties. The radiation-induced decomposition of the purine bases within DNA and related model compounds have recently been reviewed by Cadet and Berger (122).

Because of the low solubility of 2'-deoxyguanosine, approximately two orders of magnitude less than that of 2'-deoxyadenosine, we decided to use the latter purine nucleoside in our study of the direct effects. In the frozen aqueous solution system and using our sample size and dose rate, a saturated solution of 2'-deoxyguanosine would have to be irradiated for approximately three years continuously before the yields of the major products would be sufficient for their chromatographic detection, isolation and characterisation. The alternative would be to use the 5'-monophosphate which has a practical solubility, and indeed this study is envisaged, however the required use of buffered HPLC eluents led us to choose the adenine nucleoside as our first purine substrate.

3.2. The indirect effects of gamma radiation on 2'-deoxyadenosine

The reactions of the main water radiolysis products with the purine moiety of adenine nucleosides are fast processes, although the exact sites of attack remain speculative. A prime site of attack for the hydroxyl radical is the 7,8-double bond of the imidazole ring, more specifically at the C8 position. The resulting products are 8-hydroxyadenine and 7,8-dihydro-8-hydroxyadenine (Figure 3.1.). The corresponding 2'-deoxynucleoside derivatives have also been detected and characterised. In oxygen-free aqueous solution, addition of a hydroxyl radical to the C8 position also leads to opening of the imidazole ring, and to the formation of the 4,6-diamino-5-formamidopyrimidines (adenine FAPy) (123)(124) (Figure 3.2).
Figure 3.1: 8-Hydroxyadenine

Figure 3.2: Formation of 4,6-diamino-5-formamido pyrimidine.
The reaction of the solvated electron with the base moiety occurs at almost the diffusion controlled rate (125) followed by rapid proton transfer from water. All attempts to isolate any of the expected diamagnetic products have so far been unsuccessful, suggesting that either these products are not formed or that they are unstable.

The remaining known products of the indirect effects of gamma radiation on 2'-deoxyadenosine involve the formation of radicals centred in the osidic moiety. Hydrogen abstraction at C5' appears to be an important process, and the resulting radical was first observed by Alexander and Franklin (126). The main reaction undergone by this radical is intramolecular attack at C8 of the base moiety resulting in cyclisation. This reaction appeared to be stereospecific in that for 2'-deoxyadenosine only the 5'R epimer was observed (68) and the 8,5'-cycloadenosine 5'-monophosphate was shown by X-ray crystallography (127) to have the 5'S configuration. Recent studies have shown that for adenosine-5'-monophosphate (128) both the 5'R and 5'S epimers are formed. It has been shown that the relative yields of each isomer depend upon the pH and on steric considerations. Neutral pH conditions favour the 5'S configuration for the formation of 5',8-cycloadenosine-5'-monophosphate, however for polyadenosine the strand conformation is such that the 5'R conformation predominates by a factor of 2.5 (129). Interestingly, the yield of 5',8-cyclo-2'-deoxyadenosine in double-stranded DNA was shown to be two to three times higher than in single-stranded DNA (130).

Carbon centred radicals at other sites in the sugar are evoked in nucleosides demonstrating rearrangement of the osidic moiety yielding the α-anomer of the furanoid and the α- and β-anomers of the pyranoid forms (118). The formation of these products was more specifically linked to the formation of a radical centred at the 4' position followed by opening of the Cl'-O bond of the 2'-deoxyribose moiety probably via a Schiff's base type intermediate before ring reclosure through the 4'-OH (furanoid) or 5'-OH (pyranoid) groups (see Figure 2.7).

There have been no reports to date dealing with the detection of radiation-induced adenine modifications in cellular DNA. Recently, Kasai et al (131) claimed to have detected the guanine analogue of 8-hydroxyadenine in liver cells obtained from whole-body gamma-irradiated mice, and provided
evidence for its repair. 8-Hydroxyadenine and the adenine FAPy products have been detected in gamma irradiated native E. coli DNA (132), the latter products having been shown to be excised by an E. coli derived formamidopyrimidine-DNA glycosylase from polydeoxyadenylic acid irradiated with gamma rays in oxygen-free aqueous solution (133). This enzyme, reported by Chetsanga and Lindahl (134) and shown to also be formed in mammalian cells, was shown to excise the imidazole ring opened products of 7-methylguanine from alkylated DNA. The apparent presence of a gene coding for the repair of this lesion is itself evidence for the formation of FAPy lesions in vivo.

3.3. Radical mechanisms in adenine radiation chemistry

3.3.1. The adenine π-cation

For the adenine π-cation, significant unpaired electron density is expected at all ring positions, although N1, N3, C5, C8 and N9 might be expected to be favored since we know that the C6 position is favoured for cytosine and uracil. Deprotonation will occur at N9 or at N6' (the exocyclic amino group). Where the base is substituted at the N9 position, deprotonation may also occur from the C1' position. Anion addition may also return the parent charge state and will occur at C8.

3.3.2. The adenine π-anion

Protonation of the adenine π-anion will occur primarily at the C8 or C2 positions, although addition to the N3 position has not been ruled out. Gregoli et al (135) showed that irradiation of the disodium salt of 5'-AMP in frozen aqueous solution yields the C8 or C2 hydrogen adduct radical. In studies on crystalline samples, the preference for either of these positions would appear to depend on the crystal geometry and the intermolecular hydrogen bonding. Crystals of 9-methyladenine show C8 addition radicals only. Here N3 is not hydrogen bonded whereas N1 and N7 are occupied in a H-bonding cycle via two N6-H bonds. In crystals of 2'-deoxyadenosine monohydrate, both C8 and C2 adduct radicals are formed in comparable amounts. C8 addition radicals may be converted in most cases to the C2 addition radical by irradiation at 77K with UV light of 360-400 nm. Annealing to 300-400K results in the reverse conversion (136)(137).
3.4. The chromatographic isolation of the products resulting from the
direct effect of gamma radiation on 2'-deoxyadenosine

The analytical approach used in the studies of the direct effects on
thymidine and 2'-deoxycytidine may also readily be applied to the study of
2'-deoxyadenosine. Since 2'-deoxyadenosine is better retained on the reversed
phase column than thymidine, the initial fractionation is made using a solvent
of lower polarity, that is water containing 10 % methanol. The fractions that
are eluted late using this system are rechromatographed directly using the
silicagel : Solvent II system. Those which are eluted fairly early are rechro-
mamatographed on the reversed phase column using a solvent of higher polarity,
i.e. of lower methanol content, in order to increase the resolution of the
RPHPLC fractionation before progressing onto silicagel HPLC. As usual, each
fraction is checked by two-dimensional silicagel t.l.c. using Solvents I and
II. All nucleosidic products may be readily located on the plate using the
cysteine : H$_2$SO$_4$ (aq.) solution. Table III.1 lists the products isolated
during this study.

3.5. Characterisation of the product resulting from the direct effects of
gamma radiation on 2'-deoxyadenosine

All of the products isolated during this study are known products of the
indirect effects of gamma radiation on 2'-deoxyadenosine, with the exception
of the 5'S isomer of 8,5'-cyclo-2'-deoxyadenosine, and have been well documen-
ted. Their identification rests on the comparison of the $^1$H NMR data obtained
for the products isolated with those of the authentic samples published in the
literature. For the latter compound, it may be synthesised radiolytically by
irradiating 8-bromo-2'-deoxyadenosine in aqueous solution whereupon both the
5'R and 5'S isomers of the cyclonucleoside are formed (138). Comparison of the
spectral data obtained for the product isolated in the present study and that
synthesised radiolytically showed them to be the same.

3.5.1. Structural assignment of the 5'S epimer of 5', 8-cyclo-
2'-deoxyadenosine

It should firstly be noted that the 5'R and the supposed 5'S epimers of
5',8-cyclo-2'-deoxyadenosine obtained from the direct effects of gamma radia-
tion on 2'-deoxyadenosine and those obtained from irradiation of
**TABLE III.1**

1. 9-(2'-DEOXY- α-D-ERYTHROPENTAFURANOSYL)-ADENINE  
2. 9-(2'-DEOXY- β-D-ERYTHROPENTAPYRANOSYL)-ADENINE  
3. 9-(2'-DEOXY- α-D-ERYTHROPENTAPYRANOSYL)-ADENINE  
4. 5'R 5',8-CYCLO-2'-DEOXYADENOSINE  
5. 5'S 5',8-CYCLO-2'-DEOXYADENOSINE  
6. 8-HYDROXY-2'-DEOXYADENOSINE  
7. ADENINE  
8. 2-DEOXY-D-RIBONO-1,4-LACTONE

---

**TABLE III.2**

'H NMR DATA FOR THE 5',8-CYCLO-2'-DEOXYADENOSINE.

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<th>5'S EPIMER</th>
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</table>

a - chemical shifts given with respect to TSP internal standard, accuracy +0.01 ppm.

b - signals are masked by HDO peak (4.81 ppm).
8-bromo-2'-deoxyadenosine in oxygen-free aqueous solution gave identical $^1$H NMR and mass spectrometric data. The data obtained for the 5'R epimer matched those reported in the literature (118). Although the $^1$H NMR characteristics for 5',8-cycloadenosine-5'-monophosphate have been published (127), those obtained for the 5'S diastereoisomer of the 5',8-cyclo-2'-deoxynucleoside have yet to be reported and will be discussed here.

The $^1$H NMR data for both isomers are presented in Table III.2. The 5'S epimer shows similar $^1$H NMR characteristics to the 5'R isomer with slight differences which distinguish it and which provide evidence for the 5'S configuration. Both spectra demonstrate the presence of only one aromatic proton signal downfield designated as the H2 proton. We note also the lack of one further proton compared to the parent 2'-deoxynucleoside, and in particular the ABX system representing the exocyclic hydroxymethyl group. Two characteristics distinguish the isomers and allow the assignment of the absolute configurations at C5'. As pointed out by Otter et al (69) in their discussion of the conformations of the 5',6-cyclouridines, a small 4'5' coupling constant is expected for the 5'R epimer, the corresponding dihedral angle approaching 90° (see section 5.3) and indeed we observe 1.2 Hz for the isomer shown to be formed by the indirect effects. For the 5'S configuration, a Dreiding model shows these protons to be practically eclipsed and we would expect a coupling constant of 6-7 Hz (found 6.2 Hz). For the adenosine monophosphate analogue, a $J(4'5')$ value of 5.9 Hz was reported, the slight reduction in the magnitude of this coupling being expected from the proximity of the electronegative phosphate group. In D$_2$O we are unable to observe H3' and H4' signals at room temperature being isochronic with the HDO signal having a chemical shift relative to TSP of 4.81 ppm. The fact that the H3' signal is necessarily shifted downfield compared to that of the 5'R epimer (4.51 ppm) is consistent with the configurations assigned since for the former, the 5'-hydroxyl group is in closer proximity to the H3' proton.

The FAB mass spectrum confirms the expected molecular mass of 249 amu, corresponding to the parent nucleoside less two atomic mass units, however it provides little structural information from molecular ion fragmentations. The electron impact spectra however indicate distinct differences. The principal fragmentations of both epimers of 5',8-cyclo-2'-deoxyadenosine are outlined in Figure 3.3. The EI mass spectrum of each isomer is shown in Figure 3.4 where it is evident that the fragmentation patterns are the same but that the
Figure 3.3: EI mass spectrometry fragmentation patterns for 5',8-cyclo-2'-deoxyadenosine.
Figure 3.4: Electron impact mass spectra for the 5R' (a) and 5'S (b) epimers of 5',8-cyclo-2'-deoxyadenosine.
relative abundances differ considerably. For the 5'S epimer the m/z 135 fragment corresponding to the adenine molecular ion is the base peak ion. That of the 5'R epimer is m/z 165 resulting from release of the osidic moiety leaving the C5' carbon attached to the base fragment.

3.6. The gamma irradiation of 2'-deoxyadenosine in frozen D₂O solution

As discussed below, the formation of certain products generally evokes mechanisms involving hydrogen abstraction, most notably in the osidic moiety prior to rearrangement of the furanose to give either the α-furanoid form or the α- and β-pyranoid forms of the nucleoside. In order to test the implication of certain sugar centred radicals in the formation of these products and to determine whether, as in the case of thymidine, hydration water plays an important role as a proton donor under the conditions used, 2'-deoxyadenosine was irradiated in frozen deuterium oxide solution.

All products were isolated and analysed by ¹H NMR spectrometry. They were shown to contain a complete set of osidic protons, indeed the spectra were identical to those obtained during the original study in all respects.

3.7. The products resulting from the direct effects of gamma radiation on 2'-deoxyadenosine

The discussion of the likely mechanisms of the formation of the 2'-deoxyadenosine degradation products will as usual be grouped according to the type of mechanism evoked.

3.7.1. Products linked to the formation of the adenine π-cation

3.7.1.1. Adenine

Although the rearrangement of the osidic moiety may be linked to the formation of radicals centred at several sites in the sugar, the scission of the N-glycosidic bond to liberate adenine implies more specifically the C1'-centered radical. This may result from the deprotonation of the radical π-cation at the C1' position, or alternatively from a local indirect effect involving hydroxyl radicals formed in the hydration layer or even proton
abstraction by a neighbouring substrate radical. The release of adenine is quantitatively the most important process and is accompanied by the formation of significant quantities of 2-deoxy-D-ribo-n-1,4-lactone.

3.7.1.2. 8-hydroxy-2'-deoxyadenosine

The only compound implying anion addition to the adenine radical cation is 8-hydroxy-2'-deoxyadenosine, existing mostly in its carbonyl tautomeric form. We might also explain the formation of this compound by an indirect effect involving hydroxyl radical attack at C8. An interesting observation is the lack of any of the other products associated with the C8-OH adduct radical, namely 8-hydroxy-7,8-dihydro-2'-deoxyadenosine and 4,6-diamino-5-formamidopyrimidine. Possibly, the low temperature at which the OH-adduct radical is formed allows hydrogen atom transfer from C8 to reform the 7,8-double bond in preference to ring opening or hydrogen addition to the 8-hydroxy-7,8-dihydro-2'-deoxyadenosinyl radical.

3.7.2. Products linked to the formation of radicals centred in the osidic moiety

3.7.2.1. Sugar rearrangement products

Three of the quantitatively most important products result from the opening of the furanose ring with rearrangement. In all cases the radical thought to be responsible for ring opening is that centred at C4' (118)(119). For the \( \alpha \)-furanoid form, simple epimerisation occurs at C1' before ring reclosure between C1' and O4'. For the \( \alpha \)- and \( \beta \)-pyranoid forms, ring reclosure occurs via the C5' hydroxyl group. The results presented above on the direct effects of gamma radiation on 2'-deoxyadenosine in frozen D\(_2\)O solution may be interpreted in two ways. Firstly, we might continue to favour the proposal of the formation of a C4' centred radical. In this case, after rearrangement of the osidic moiety, reduction of the C4' centre does not occur via hydrogen transfer from a hydration water molecule. This necessarily means that transfer occurs from a site within the nucleoside and furthermore not from a heteroatom. As discussed in Chapter I concerning the formation of the dThd(\( \alpha \)-6)hdThd adducts, hydrogen atom transfer may well occur from the C5' position. If all processes involving hydration water are in fact proton transfers, and not hydrogen atom transfers, the fact that the C4' radical is
reducing would rule out the necessary electron transfer to this radical before protonation. In such cases it may be that the source of the hydrogen atom added to C4' be a non-exchangeable one, probably from the C5' position or even C8 during the formation of 8-hydroxy-2'-deoxyadenosine. This hypothesis does not contradict the results observed for thymidine since, for the formation of 5,6-dihydrothymidine, the initial step is the protonation of the initial radical π-anion at C6. The resulting 5-yl is oxidising and we may therefore reasonably propose a reduction in two steps, firstly by electron transfer to C5' to form the carbanion followed by again protonation involving a hydration water molecule.

The results may however deny the involvement of the C4' radical in the formation of these products. We might suggest instead a weakening of the already weak N-glycosidic bond relative to the pyrimidine nucleosides by direct excitation of the molecule, leading to subsequent ring opening.

3.7.2.2. 8,5'-cyclo-2'-deoxyadenosine

As mentioned in the above discussion on the indirect effects of gamma radiation on adenine nucleosides, the radical responsible for the cyclisation reaction leading to the formation of 8,5'-cyclo-2'-deoxyadenosine results from initial hydrogen abstraction from the C5' position with subsequent attack at C8 of the base (Figure 3.5). We note that cyclisation in this case lacks stereospecificity in agreement with recent findings and that both the 5'R and 5'S epimers are formed in comparable amounts, the 5'R diastereomer being slightly favoured.
3.8. A comparison between the direct effects of gamma radiation on 2'-deoxyadenosine and pyrimidine nucleosides

A clear distinction that we may draw between the two modes of degradation is the relative importance of products resulting from the formation of radicals centred in the sugar moiety for the purine nucleoside. This may be accounted for in two ways. Firstly, it is likely that base centred radicals for purine and pyrimidine nucleosides be formed with similar G values, since it is known that electron addition and subsequent protonation are fast processes, and the observation of only one product resulting from chemical modification of the base, that is 8-hydroxy-2'-deoxyadenosine, in a yield comparable to those of products implying the formation of furanose centred radicals, suggests strongly the occurrence of processes which regenerate the parent purine structure. Secondly, radical centres in the sugar moiety have been shown to be formed for both substrates (vide infra). For thymidine, the 4'-centred radical results in the formation of the α-lyxo form and possibly in base release. For 2'-deoxyadenosine, the same radical appears to give rise to ring opening between the Cl' and O4' atoms yielding the furanoid and pyranoid forms of the parent nucleoside. This may be related to the weaker bond strength of the N-glycosidic link of purine compared to those of the pyrimidine nucleosides. The relative importance of sugar radical induced damage for 2'-deoxyadenosine compared to thymidine and 2'-deoxycytidine may simply reflect the importance of restitution processes for primary base radicals of the former.
CHAPTER IV

THE DIRECT EFFECTS OF 254 NM LIGHT ON THYMIDINE

4.1. Introduction

Although the study of the direct effects of ultra-violet light on thymidine represents an interesting study in itself, both from an analytical and biological point of view, the initial motive for its incorporation into the present study of the direct effects of gamma radiation on this nucleoside was to make a comparison of the products formed by radical events and those associated with essentially excitation processes. As will be discussed, we have been able to attribute the formation of certain products of gamma irradiation to excitation processes and, owing to the increased yields of these products afforded by irradiating with UV light, we were able to use this technique to probe the mechanisms involved in their formation.

Under the effects of ionising radiation, excitation may occur in three ways. The first is discussed in the first chapter and involves a Cerenkov emission. Secondly, excitation may occur via direct absorption of the energy of the incident photon which may be distributed throughout the molecule leading to vibrational and rotational as well as electronic excitation which, although important, is insufficient for electron dissociation to occur. Finally, the most probable mechanism would involve return of an electron to a pristine radical \( \pi \)-cation if the site of electron capture is not far from the parent molecule. Indeed, Graslund et al (139) have observed a weak thermoluminescence in irradiated DNA in the temperature range 77-120 K attributed to electron transfer from a thymine \( \pi \)-anion to the guanine \( \pi \)-cation.

Once the aim of this study had been achieved, we became interested in the determination of the structures of certain fluorescent products apparently unique to UV irradiation. The Pyr(6-4)Pyo adducts appear to be highly unstable in aqueous solution giving as many as ten autodegradation products. We have attempted to isolate these products in sufficient quantity to allow a structural analysis. They may, like the Pyr(6-4)Pyo adducts themselves, be of biological importance. Also, despite the numerous studies carried out on these products, a detailed spectroscopic study of the nucleoside analogue does not appear to have been made.
For a comparison of the direct effects of both ionising and 254 nm UV light on thymidine, we used identical conditions for both studies in order for the comparison to be valid. However, for the preparation of the spore photoproducts in large quantity, and also the dThd(6-4)dPyo adducts, the thin film state appeared more appropriate.

4.2. Irradiation of thymidine in frozen aqueous solution with 254 nm UV light

The conditions of irradiation were, as far as possible, kept identical to those used during the study of the direct effects of gamma radiation, that is a similar concentration and a temperature of 196 K. For reasons of penetration of the UV light, the sample was irradiated as a layer of quite large surface area (approx. 600 cm²) and of 5 mm thickness. For practical reasons, the sample was not degassed, however the presence of oxygen is not expected to play a significant role in the degradation processes.

4.2.1. Isolation of the products resulting from the irradiation of thymidine in frozen solution

The yields of the major products of UV irradiation are quite low and necessitate the same experimental approach as that used in the gamma study. The major proportion of the undegraded thymidine was removed by precipitation from hot ethanol. The resulting mixture was injected on to a reversed phase preparative column and the fractions obtained rechromatographed on analytical reversed phase and silicagel HPLC columns in the usual way. The products isolated during this study are listed in Table IV.1.

4.2.2. Product identification

4.2.2.1. The cyclobutane dimers

The cyclobutane dimers of thymidine have been characterized in detail in the literature (61) and were identified in the present study by simple comparison of their $^1$H NMR characteristics with literature data. The products also underwent photosplitting of the cyclobutane ring when exposed to 254 nm UV light to give thymidine, a characteristic reaction of the cyclobutane dimers.
TABLE IV.1

PRODUCTS RESULTING FROM THE FAR UV IRRADIATION OF THYMIDINE IN FROZEN AQUEOUS SOLUTION

1. Cyclobutidithymidine (cs, ca+, ca-, ts-, ta) (61).
2. dThd(α-5)hdThd (5R, 5S).
3. dThd(6-4)dPyo I.
4. dThd(6-4)dPyo II.

TABLE IV.2

$^1$H NMR DATA (a) FOR NEW dThd(6-4)dPyo ADDUCTS

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<thead>
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<th></th>
<th>dThd(6-4)dPyo I</th>
<th>dThd(6-4)dPyo II</th>
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<tbody>
<tr>
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</table>

a - Chemical shifts are given in ppm against TPS internal standard. Coupling constants are in Hz.
4.2.2.2. The spore photoproduct

Both isomers of the spore photoproduct were isolated, the structural assignments of which were made on the basis of FAB mass spectrometry and $^1$H NMR data. The analysis of these compounds, which have not been characterised to date as the nucleoside dimers, is described in Chapter I.

4.2.2.3. The dThd(6-4)dPyo adducts

These products are well-known photoproducts, and have been characterised as the free base dimer (140) and as the dinucleotide monophosphate (141). To our knowledge these products have not been characterised as the dimeric nucleoside. The molecules have been shown to be dimeric by FAB-MS indicating a mass of 484 amu. Plasma desorption mass spectrometry (PDMS) yielded evidence of a dimer having a molecular weight equivalent to a thymidine dimer having gained a molecule of water. The two mass spectrometric techniques would appear to suggest two different structures (Figure 4.1). Under the conditions of the FAB technique, it was possible that the molecular ion dehydrate in the source giving an apparent molecular mass lower than the actual mass. We recorded a $^1$H NMR spectrum in DMSO-$d_6$ and demonstrated the presence of only one imide proton confirming the FAB-MS data. Both isomers are fluorescent, and show an absorption maximum at 320 nm.

The $^1$H NMR spectra are shown in Figures 4.2 and 4.3, and the spectral data obtained listed in Table IV.2. Signal assignments were made by selective decoupling experiments and the spectrum of the first isomer has been iterated using the LAOCOON III program. We shall discuss here the general $^1$H NMR features supporting the proposed structure. Chemical shifts given in brackets refer to the second isomer, assigned arbitrarily since it is that eluted last on the reversed phase HPLC system.

Each isomer shows the presence of only one vinyl proton downfield at 8.25 ppm (8.32 ppm) and the corresponding methyl group signal appears at 2.25 ppm (2.10 ppm) indicating the expected intact 5,6-double bond for one of the nucleoside residues. There is another methyl signal at 1.74 ppm (1.63 ppm) observed as a singlet suggesting the lack of a proton at C5 of the saturated residue. The chemical shift of this signal is downfield of that expected when attached to a tertiary or quarternary carbon and might indicate the presence
Figure 4.1: The proposed structures of the dThd(6-4)dPyo photoadducts suggested by (a) PDMS and (b) FABMS.

TABLE IV.3

PRODUCTS RESULTING FROM THE FAR UV IRRADIATION OF THYMIDINE AS A THIN FILM.

1. (5R) and (5S) 5,6-dihydrothymidine.
2. Cyclobutidithymidine (cs, ca+, ca-, ts-, ta) (61).
3. dThd(α-5)hdThd (5R, 5S).
4. dThd(6-4)dPyo I.
5. dThd(6-4)dPyo II.
6. dThd(α-6)hdThd I.
7. dThd(6-4)dPyo degradation product.
Figure 4.2: 400 MHz $^1$H spectrum in D$_2$O of DTHAD(6-4)Dpyo I.

Internal reference TSP.
Figure 4.3: 400 MHz 1H spectrum in D2O of dThd(6-4)dPyo II.

Internal reference TSP.

- 4
of an electronegative atom at this position, consistent with a 5-OH group. The observation of the H6 signal of the saturated residue in the same chemical shift region as that of the thymidine diols would indicate firstly, as a singlet, that there are no geminal or vicinal protons and hence that the internucleoside bridge occurs at this position, and that the H6 proton is highly deshielded by the substituent at C6 consistent with the proximity of the aromatic system represented by the unsaturated base ring. The remaining proton signals present chemical shifts and coupling patterns expected of two complete furanose residues.

4.3. The irradiation of thymidine as a thin film with 254 nm UV light

The irradiation of thymidine as a thin film provides another system for the study of the direct effects of far UV light and, as will be described, yields interesting additional information to the study of the frozen aqueous system. The characterisation of the products isolated in this study are described elsewhere in this work. Table IV.3 lists the products found.

4.3.1. The isolation of the products formed by the irradiation of thymidine with 254 nm light as a thin film

The usual methodology is used, i.e. precipitation of undegraded thymidine from hot ethanol, crude fractionation on a reversed phase preparative HPLC column followed by reversed phase and silicagel analytical HPLC.

4.4. Mechanistic studies on the direct effects of 254 nm radiation on thymidine

Ultra-violet radiation may generally induce chemical reaction in three ways. Firstly, the radiation may induce homolytic bond scission to form radical species which, being highly reactive, may either decompose releasing a smaller or more stable radical and a diamagnetic molecule, or may react with other molecules in its vicinity, such as the radical propagation in polymerisation processes. Secondly, the absorbed energy may simply provide an activation energy for a given reaction, raising the energy of one or both of the reactants sufficiently to overcome the reaction energy barrier. Finally, the absorbed photon may be of sufficient energy to eject an electron from its orbital in an ionising process. This is frequently encountered with laser sources of UV radiation where a single photon has insufficient energy to
ionise a particular molecule, but where in this case the photons are of such an intensity that absorption of a second photon may occur before the excited molecule has time to dissipate the energy transferred by the first. These are known as biphotonic processes. These processes clearly infer the involvement of several possible mechanisms in the formation of a particular product, and we must therefore devise experiments which may demonstrate or rule out the involvement of a suspected process. For the formation of the spore photoproduct, as in the case of its structural isomer, the dThd(α-6)hdThd adducts, formed during the gamma irradiation of thymidine in frozen aqueous solution, we might envisage both a consecutive mechanism and a concerted one. We might suggest the addition of a hydrogen atom to C6 to form 5-yl radical and also the formation of methylene radical via hydrogen atom loss from the methyl group of a thymidine molecule with simple combination of these radicals, or alternatively a concerted mechanism involving simultaneous transfer of a hydrogen atom from the methyl group of one nucleoside molecule to C6 of the other and bond formation between the methyl carbon and C5 of the eventual saturated residue.

4.4.1. Irradiation of thymidine in frozen D2O solution with 254 nm UV light

In order to determine whether radical intermediates are involved in the formation of the spore photoproducts, thymidine was irradiated in frozen D2O solution. The proposed 5-yl radical may be formed by addition of a hydrogen atom to the 6-position of thymidine possibly from a hydration water molecule. In this case we would expect incorporation of deuterium at C6 of the spore photoproduct. All of the major photoproducts listed in Section 4.2 were isolated and analysed by 1H NMR spectrometry. The results showed that no molecules undergo incorporation of deuterium at any position and the spectra are identical to those obtained under the conditions of the initial study.

4.4.2. Irradiation of thymidine deuterated at the methyl group as a thin film with 254 nm UV light

This experiment represented a powerful probe in the determination of the mechanism involved in the formation of the spore photoproduct. It provides an unambiguous result concerning the possibility of proton transfer from the methyl group of one nucleoside residue to the C6 position of the other thymidine residue. Figure 4.4 shows the CH2 group signals at position C6 of
Figure 4.4: C6 methylene 400 MHz $^1$H NMR signals for dThd($\alpha$-5)hdThd II from the 254 nm UV irradiation of thymidine and CD$_3$-thymidine as thin films.
the saturated base ring in the 400 MHz $^1H$ NMR spectra of the second isomer of the spore photoproduct isolated from the far UV irradiation of thymidine as a thin film non-deuterated at the methyl group, and from the irradiation of the deuterated sample. It is clear that deuterium transfer has occurred to a large extent although not at 100%. Quantitation by FAB mass spectrometry and integration of the appropriate parts of the $^1H$ NMR signal of each molecule—deuterated or non-deuterated at the C6 position—puts deuterium transfer at approximately 65%.

The results suggest that under the thin film conditions, two mechanisms are involved. One mechanism, and clearly quantitatively the most important, involves direct transfer of a methyl proton to the C6 position of the eventual saturated base residue in a purely concerted mechanism. The second would apparently involve hydrogen atom transfer from a position other than the methyl group to H6 to give the 5-yl radical. Such a radical must undergo combination with the methyl carbon of another nucleoside molecule. This may theoretically occur in two ways, namely loss of a hydrogen at this position to give the methylene radical which combines directly with the 5-yl radical, or by attack at the methyl carbon with concomitant displacement of a methyl proton. We cannot distinguish between these two possibilities.

4.5. A comparison of the direct effects of far UV light on thymidine irradiated in frozen aqueous solution or as a thin film

It is clear, both from the point of view of the quantitative importance of the products common to both conditions and from the nature of the products formed where these are different in the two cases, that the state of the substrate plays an important role in product formation under the direct effects of 254 nm light on thymidine. In order to better define these differences, we need to irradiate thymidine deuterated at the methyl group in frozen aqueous solution in order to determine whether there are two mechanisms at work in the formation of the spore photoproducts under these conditions. Although irradiation of thymidine in frozen $D_2O$ solution revealed no incorporation of deuterium, this does not rule out the intermediate formation of the 5-yl radical. Hydration water is likely to take part in protonation processes rather than hydrogen atom transfer which implies photoionisation of the thymidine molecule. Under the conditions of frozen aqueous solutions, biphotonic ionisation processes are unlikely and therefore the lack of any role of hydration water is not a surprising result. However, it may be possible that hydrogen atom
transfer from a non-exchangeable proton site may occur. An indication that this is not the case and that under these conditions the process is 100% concerted was provided by the gamma radiation of thymidine deuterated at the methyl group in the frozen aqueous state. It was observed that under conditions of predominant ionisation, the mechanism was concerted with apparently no contribution from a consecutive process. The result was unfortunately not unambiguous since the isomer isolated was that having isochronous C6 protons which did not allow the detection of a geminal coupling of the type shown in Figure 4.4 where deuterated thymidine was irradiated with far UV as a thin film. Nonetheless, the integral of the appropriate singlet \(^1\)H NMR signal and the FAB mass spectrum indicated quantitative deuteration at C6 of the saturation base.

It is interesting that hydrogen atom formation appears to occur in the irradiation of thymidine as a thin film, but not in the frozen state. The evidence for this is quite clear. When irradiated as a thin film, thymidine gives rise to the formation of 5,6-dihydrothymidine as well as the structural isomer of the spore photoproducts, the dThd(α-6)hdThd adducts shown to be formed via the direct effects of gamma radiation on thymidine irradiated in frozen aqueous solution and where the addition of a hydrogen atom to the C5 position was suggested to be the mechanism involved. In addition, the spore photoproducts formed in thymidine irradiated as a thin film were shown to be formed by a consecutive as well as the predominant concerted mechanism implying hydrogen atom addition to C6. When thymidine was UV-irradiated in frozen aqueous solution, neither the 5,6-dihydrothymidines nor the dThd(α-6)hdThd adducts were detected.

The other major difference in the effects of far UV light on thymidine irradiated in the frozen state and as a thin film is the relative yields of those products common to both conditions. In frozen aqueous solution, the major products are the cyclobutidithymidines with a distinctly lower formation of the dThd(6-4)dPyo adducts, and even more so for the spore photoproducts. As a thin film, the major products are the spore photoproducts, followed by the cyclobutyl dimers and finally the dThd(6-4)Pyo adducts. This tendency towards an increasing relative formation of the spore photoproducts compared to the yields of the cyclobutyl dimers with decreasing degree of hydration has also been observed for DNA as described in the Introduction to this thesis.
4.6. A comparison of the direct effects of 254 nm UV and gamma radiations on thymidine

As hoped, the study of the direct effects of 254 nm UV light on thymidine has provided an interesting insight into the processes occurring during the gamma irradiation of thymidine in frozen aqueous solution. The cyclobutyl dimers and the spore photoproducts are apparently products common to both irradiation types when carried out in the frozen state. In each case, the relative yields of these products are apparently the same judging by the HPLC chromatograms which might suggest that the processes leading to their formation are the same. In addition, the mechanistic studies carried out in both studies on the formation of the spore photoproducts, although involving a different approach in each case, seem to support this view.

The formation of the dThd(α-6)hdThd adduct via UV irradiation only under conditions where hydrogen atoms are produced in significant quantities supports the conclusion of the mechanistic study of their formation under the effects of gamma radiation, that the initial step involves the addition of a hydrogen atom at C5 of a nucleoside followed by subsequent combination with a 5-(2'-deoxyuridilyl)methyl (thymidyl) radical.

The most noteworthy difference between the UV and gamma studies is the observed lack of the dThd(6-4)dPyo adduct during the gamma irradiation of thymidine even though this is quantitatively more important than the spore photoproducts, themselves isolated during the gamma study, when irradiated with 254 nm light under the same conditions. As discussed later in this chapter, the dThd(6-4)dPyo adducts are unstable in aqueous solution, and we may therefore forward the explanation that they decompose during the isolation process which, in the case of the gamma irradiation study is longer owing to the increased complexity of the degradation product mixture. However, the dThd(6-4)dPyo adducts have been shown to give a major decomposition product which is itself stable and which was also not isolated during the gamma experiment.

The apparent lack of formation of fluorescent adducts in the gamma irradiation study supports our view that a Cerenkov emission does not account for the major proportion of the observed yields of the cyclobutidithymidines and the spore photoproducts produced under these conditions, and suggests that the UV irradiation of thymidine in frozen aqueous solution does not imitate
exactly the excitation processes occurring under exposure to ionising radiation.

4.7. The autodegradation of the dThd(6-4)dPyo adducts

The instability of the dThd(6-4)dPyo adducts in aqueous solution has been remarked upon during other studies carried out by our team, however this observation has not been published and no attempt has been made so far to identify the products of this autodegradation and to delineate the mechanisms involved. The study is not complete since the mixture of products is complicated and some are formed in small quantities.

There is one degradation product which is quantitively the most important and which is apparently stable, undergoing no further degradation in aqueous solution. This product has been isolated and analysed by UV absorbance, $^1$H NMR and FAB mass spectrometries.

This product was shown to be dimeric by FAB mass spectrometry which gave a molecular mass of 484 amu, identical to the parent molecule. This ruled out the possibility of a simple loss of water from the saturated base residue of the parent dimer. The UV absorbance spectrum shows the product to be fluorescent showing an absorbance maximum at 319 nm, however it differs from the parent molecule due to the presence of an additional absorbance band at 272 nm, which suggested to us the formation of an extra double bond conjugated with a base carbonyl group. The $^1$H NMR spectrum does not provide much supplementary information (Table IV.4). It confirms that the molecule is dimeric with two complete sets of osidic protons and two methyl groups. The NMR features do not differ much from the parent molecule.

**TABLE IV.4**

| $^1$H NMR DATA FOR THE DTHD(6-4)DPYO DEGRADATION PRODUCT |
|-----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| $\delta'$      | $\delta''$ | $\delta''$ | $\delta'''$ | $\delta''''$ | $\delta''''''$ | $\delta'''''''$ | $\delta'''''''$ | $\delta'''''''$ | $\delta'''''''$ |
| A               | 6.05  | 2.04  | 1.91  | 4.37  | 3.90  | 3.78  | 3.72  | 2.23  | 1.64  | 8.33  | 5.74  |
| B               | 6.19  | 2.62  | 2.29  | 4.41  | 4.16  | 3.90  | 3.78  |       |       |       |       |
| $1'2''$         | $1'2''$ | $2'2''$ | $2'3''$ | $2'3''$ | $3'4''$ | $4'5''$ | $4'5''$ | $5'5''$ |       |       |       |
| A               | 7.8   | 6.4   | -13.9 | 6.7   | 3.6   | 3.4   | 3.9   | 4.6   | -12.4 |       |       |
| B               | 6.6   | 5.5   | -14.2 | 4.9   | 6.6   | 4.2   | 3.3   | 4.8   | -12.7 |       |       |
We still have one vinyl proton at 8.24 ppm presenting a tight quartet through long-range coupling to the methyl group at 2.14 ppm. We have a second methyl group seen as a singlet at 1.50 ppm attached to a carbon again carrying no protons, and the H6 proton belonging to the saturated base residue is seen as a singlet at 5.65 ppm indicating that it is \( \alpha \) to a highly electronegative group. The similarity between the \( ^1 \text{H} \) NMR spectrum of this degradation product and those of the two isomers of the dThd(6-4)dPyo adduct suggests that the former is either a structural isomer of the latter two compounds or that the protons added are exchangeable. An interesting possibility exists in that we might envisage the addition of a molecule of water to the N3-C4 double bond to give that structure suggested by the plasma desorption mass spectrum. It may be that the peak seen in the latter spectrum results from the presence of the degradation product as an impurity. Under the FAB mass spectrometric conditions, the molecule might effectively dehydrate to indicate a mass lower than the real one. We shall attempt to obtain this compound in greater amounts in order to effect a \( ^1 \text{H} \) NMR spectrum in an aprotic solvent to determine the number of imide protons.
5.1. The conformational properties of nucleic acids.

It is well-established that the furanose ring is not planar but puckered, the many possible conformations being best described by the concept of pseudorotation introduced by Kilpatrick et al. (142) where the amplitude of the puckering rotates round the ring. In cyclopentane this rotation is free; in contrast however, the presence of substituents on the furanose ring results in the existence of potential energy barriers between preferred conformations.

For nucleic acid components, a particular conformation is not only defined by the degree and type of puckering of the five-membered ring, but also by the relative orientations of exocyclic substituents, namely those of the base residue at C1' and the low energy rotamers of the exocyclic hydroxymethyl group at C4'.

In solution at ambient temperature interconversions between all possible conformations are fast on the NMR time-scale and thus an NMR spectrum represents the characteristics of a time-averaged conformation related to the equilibrium populations of each conformer. The conformational information yielded by a $^1$H NMR spectrum refers to this "averaged conformer". As discussed below, the solution NMR spectrum of a particular nucleoside derivative allows the quantitative determination of the possible conformations, that is their contribution to the averaged conformation.

5.1.1. 2'-Deoxyribofuranose conformation.

A useful description of the possible conformations due to ring puckering in nucleotides was introduced by Altona and Sundaralingam (143). They used a description based on the concept of pseudorotation mentioned above which allows the determination of the exact conformation of a particular furanoid ring in terms of two parameters, the phase angle of pseudorotation $P$ and the degree of puckering $\tau_m$. 
Figure 5.1

$N \ (3'-\text{endo} \ 2'-\text{exo})$  $S \ (2'-\text{endo} \ 3'-\text{exo})$

Figure 5.2: Extreme base-sugar orientations. (a) syn and (b) anti.

Figure 5.3: Stable rotamers of the exocyclic hydroxymethyl group.
The furanose ring is said to be in equilibrium between two extreme conformations (Figure 5.1). For the type N conformation classically denoted as C3' endo C2' exo, the phase angle P is arbitrarily set at 0°. Pseudorotation about 180° yields the mirror image of the original ring, now having the C2' endo C3' exo conformation, and defined as type S. When P goes from 0-360°, the molecule completes a full pseudorotational cycle and returns to its original conformation.

The degree of puckering \( r \) generally varies between 35-45° with an average of 39°. There appears to be little or no correlation between \( r \) and P (143). It has been noted that the range of experimentally found P values for 2'-deoxyribonucleosides is greater than that found for ribonucleosides suggesting that for the former the potential energy wells associated with each stable conformation are much flatter.

The above authors found (143)(144) that the value of \( J(2'3') \) is fairly independent of the sugar conformation and that \( N \langle 1'2' \rangle = S \langle 3'4' \rangle = 0 \) Hz and \( S \langle 1'2' \rangle = N \langle 3'4' \rangle = 10 \) Hz and thus that the sum \( J(1'2')+J(3'4')=10 \) Hz whatever the furanose conformation assuming that the ring has no induced rigidity. Guschlbauer and Son (145) calculated the coupling constants for all conformations for values of P=0-360° and \( r = 30-50° \). This method has recently been criticised because of certain assumptions made in the calculations, in particular that the degree of puckering in the N conformation is the same as that for the S conformation. Nevertheless, the graphs serve to demonstrate a trend and therefore the values of \( r \) for each deoxyribose residue of the new dimeric products have been determined and are listed in Table V.1.

The furanose conformation may also be described by the equilibrium populations of the C2' endo and C3' endo conformers, either using the semi-quantitative relationship \( \%C3' \text{ endo} = 10 \times J(3'4') \) or \( \%C3' \text{ endo} = 100 \times J(3'4')/J(1'2')+J(3'4') \) which follows from the arguments of Davies and Danyluk (146). The resulting values are presented in Table V.2.

5.1.2 Base orientation.

An important conformational feature of nucleotides and nucleosides is the relative orientations of the base and sugar moieties described by the torsional angle \( \chi_{CN} \) defined by Donohue and Trueblood (147) as the angle formed by
<table>
<thead>
<tr>
<th>Adduct</th>
<th>TM (°C)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>dThd(α-6)hdThd 1</td>
<td>A</td>
<td>38.0</td>
</tr>
<tr>
<td>B</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>dThd(α-6)hdThd 2</td>
<td>A</td>
<td>38.3</td>
</tr>
<tr>
<td>B</td>
<td>27.5</td>
<td></td>
</tr>
<tr>
<td>dThd(α-α)dThd</td>
<td></td>
<td>38.0</td>
</tr>
<tr>
<td>dThd(3-5)hdThd 1</td>
<td>A</td>
<td>37.4</td>
</tr>
<tr>
<td>B</td>
<td>41.6</td>
<td></td>
</tr>
<tr>
<td>dThd(3-5)hdThd 2</td>
<td>A</td>
<td>37.0</td>
</tr>
<tr>
<td>B</td>
<td>41.0</td>
<td></td>
</tr>
<tr>
<td>dThd(α-3)dThd</td>
<td>A</td>
<td>32.5</td>
</tr>
<tr>
<td>B</td>
<td>38.0</td>
<td></td>
</tr>
<tr>
<td>dThd(5'-6)hdThd 1</td>
<td>A</td>
<td>39.5</td>
</tr>
<tr>
<td>B</td>
<td>36.2</td>
<td></td>
</tr>
<tr>
<td>dThd(5'-6)hdThd 2</td>
<td>A</td>
<td>28.0</td>
</tr>
<tr>
<td>B</td>
<td>39.0</td>
<td></td>
</tr>
<tr>
<td>dThd(α-5)hdThd 1</td>
<td>A</td>
<td>38.0</td>
</tr>
<tr>
<td>B</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>dThd(α-5)hdThd 2</td>
<td>A</td>
<td>38.0</td>
</tr>
<tr>
<td>B</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>dThd(6-4)dPyo 1</td>
<td>A</td>
<td>37.9</td>
</tr>
<tr>
<td>B</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td>dThd(6-4)dPyo 2</td>
<td>A</td>
<td>36.2</td>
</tr>
<tr>
<td>B</td>
<td>33.8</td>
<td></td>
</tr>
</tbody>
</table>

(1) - determined according to Ref. 145.
### TABLE V.2

**FURANOSE CONFORMATION FOR NEW THYMIDINE ADDUCTS**

<table>
<thead>
<tr>
<th>PERCENTAGE N (3'-ENDO) CONFORMER</th>
<th>A (1)</th>
<th>A (2)</th>
<th>B (1)</th>
<th>B (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( d\text{Thd}(\alpha-6)d\text{Thd} ) I</td>
<td>39</td>
<td>37</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>( d\text{Thd}(\alpha-6)d\text{Thd} ) II</td>
<td>38</td>
<td>36</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>( d\text{Thd}(\alpha-\alpha)d\text{Thd} )</td>
<td>38</td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( d\text{Thd}(3-5)d\text{Thd} ) I</td>
<td>38</td>
<td>34</td>
<td>46</td>
<td>42</td>
</tr>
<tr>
<td>( d\text{Thd}(3-5)d\text{Thd} ) II</td>
<td>37</td>
<td>32</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>( d\text{Thd}(\alpha-3)d\text{Thd} )</td>
<td>42</td>
<td>43</td>
<td>57</td>
<td>46</td>
</tr>
<tr>
<td>( d\text{Thd}(5'-6)d\text{Thd} ) I</td>
<td>41</td>
<td>38</td>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>( d\text{Thd}(5'-6)d\text{Thd} ) II</td>
<td>20</td>
<td>19</td>
<td>41</td>
<td>36</td>
</tr>
<tr>
<td>( d\text{Thd}(\alpha-5)d\text{Thd} ) I</td>
<td>38</td>
<td>36</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td>( d\text{Thd}(\alpha-5)d\text{Thd} ) II</td>
<td>39</td>
<td>37</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>( d\text{Thd}(6-4)d\text{Pyo} ) I</td>
<td>45</td>
<td>40</td>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>( d\text{Thd}(6-4)d\text{Pyo} ) II</td>
<td>43</td>
<td>39</td>
<td>42</td>
<td>34</td>
</tr>
</tbody>
</table>

(1) Calculated according to \( %N = 10 \times \text{J}(3'4') \)

(2) Calculated according to \( %N = 100 \times \text{J}(3'4') / \text{J}(1'2') + \text{J}(3'4') \)
the trace of the plane of the sugar with the projection of the base ring when viewed from N1 to Cl'. This angle was set arbitrarily at zero when the furanose ring oxygen is anti-planar to C2 of the base and increases on turning the base plane in a clockwise direction, again looking from N1–Cl'. There are two ranges of $\chi_{\text{CN}}$ within which relatively stable conformations might be assumed, each range covering slightly more than 90°, one centred at -30° (anti range) the other centred at +150° (syn range). Put simply, conformers where the C6 of pyrimidines and C8 of purines lies over the plane of the sugar are said to be anti, and those where the C2 position lies over the furanose moiety are defined as syn (Figure 5.2). A broad range of $\chi_{\text{CN}}$ values are accessible, with the anti range occuring about eight times more frequently than the syn (148). For pyrimidine nucleosides the anti conformation is usually adopted owing to steric destabilisation of the syn conformer by interaction of the more bulky C2 carbonyl group with the endo group of the puckered furanose ring. However, pyrimidines substituted at the C6 position may lead to a preferential syn conformation as shown by George et al (149) for 6-methyl-2'-deoxyuridine.

The determination of the torsional angle for a particular nucleoside or nucleotide is not an easy matter. The most precise values come from X-ray crystallographic data, however, in the absence of a decent crystal, NMR data may be used to provide good approximate values. The methods available include relaxation time measurements, nuclear Overhauser enhancement studies (150), and chemical shift changes. Time has not permitted the former studies, we shall therefore base our discussion on the base-sugar orientations on the changes induced in the chemical shifts of certain indicator resonances. A good indication of the syn conformation is a downfield shift of the H2' signal compared to that of H2", owing to the proximity of the dipolar and anisotropic carbonyl group at C2, and an upfield shift of the H1' signal (151). Torsional angles of approximately -90° bring the C2 keto group in close proximity to the H1' proton, and we would therefore expect a downfield shift of the corresponding NMR signal. In addition, the chemical shift of the H6 signal is also expected to be sensitive to changes in $\chi_{\text{CN}}$ due to the electric field and magnetic anisotropy effects of the ether oxygen O1' (152). This is likely to lead to a downfield shift for anti conformations.
The exocyclic hydroxymethyl group at C4' of the furanose moiety may exist in three energetically favourable conformations as shown in Figure 5.3. The contributions of these low energy conformers to the time-averaged conformation depend upon the relative orientation of the base (syn or anti) and, in the case of the anti conformer, the nature of the substituent at C6. From the $^1$H NMR spectrum, we may determine these contributions from the magnitudes of the 4'5' and 4'5" coupling constants. Firstly, the contribution of the $g^+$ conformer may be obtained from the expression derived by Haasnoot et al (153), based on Karplus type considerations. The contribution of the $t(\phi)$ conformer is obtained according to Remin and Shugar (65).

\[
g^+ = 13.75 - \frac{(J(4'5')) + J(4'5'\prime\prime))}{10.05}
\]

\[
t(\phi) = \frac{J(4'5'\prime\prime) - 1.5}{10}
\]

It is assumed that the H5' signal is the downfield part of the exocyclic hydroxymethyl group signal. Once the contributions of $g^+$ and $t(\phi)$ have been calculated, that of $g^-$ may be obtained directly. These have been calculated for the new thymidine adducts and are given in Table V.3.

5.1.4. The biological importance of nucleoside conformation.

It is known that the conformation of the base and sugar residues in DNA and RNA are of great biological importance. In the native forms of double-stranded DNA, the three known forms imply particular conformations of the nucleotide building blocks. For the A form, the base residues are anti and the sugar is effectively N or 3'-endo, with a $g^+$ orientation for the exocyclic group. For the B form, the base is again N and the exocyclic group $g^+$ but this time the base orientation is syn. For the rarer Z form, 2'-deoxyguanosine is N, syn and $t(\phi)$ or $g^-$, whilst 2'-deoxycytidine is $S$, anti and $t(\phi)$ or $g^-$. 

Nucleoside diphosphates existing in a principally syn conformation have been shown to inhibit polymerisation by polynucleotide phosphorylase (154). It is interesting that homopolymers in the syn conformation were shown to bind strongly to the enzyme but restricted its polymerising activity (although the phosphorolysis was shown to occur). It was deduced that the enzyme required
**TABLE V.3**

**POPULATIONS OF THE $g^+$, t($\phi$) AND $g^-$ ROTAMERS FOR THE NEW THYMIDINE ADDUCTS**

<table>
<thead>
<tr>
<th>Adduct</th>
<th>$g^+$</th>
<th>t($\phi$)</th>
<th>$g^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dThd($\alpha - 6$)hdThd 1</td>
<td>48.3</td>
<td>21.0</td>
<td>30.7</td>
</tr>
<tr>
<td></td>
<td>39.3</td>
<td>28.0</td>
<td>32.7</td>
</tr>
<tr>
<td>dThd($\alpha - 6$)hdThd 2</td>
<td>63.1</td>
<td>27.5</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>52.6</td>
<td>30.4</td>
<td>17.0</td>
</tr>
<tr>
<td>dThd($\alpha - \alpha$)dThd</td>
<td>56.2</td>
<td>30.0</td>
<td>13.8</td>
</tr>
<tr>
<td>dThd(3-5)hdThd 1</td>
<td>42.3</td>
<td>40.0</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>51.2</td>
<td>36.0</td>
<td>12.8</td>
</tr>
<tr>
<td>dThd(3-5)hdThd 2</td>
<td>42.3</td>
<td>40.0</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>48.3</td>
<td>43.0</td>
<td>8.7</td>
</tr>
<tr>
<td>dThd($\alpha 3$)dThd</td>
<td>57.2</td>
<td>35.0</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>44.3</td>
<td>43.0</td>
<td>12.7</td>
</tr>
<tr>
<td>dThd(5'-6)hdThd 1</td>
<td>41.0</td>
<td>42.1</td>
<td>16.8</td>
</tr>
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<td>dThd(5'-6)hdThd 2</td>
<td>36.3</td>
<td>47.0</td>
<td>16.7</td>
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<tr>
<td>dThd($\alpha - 5$)hdThd 1</td>
<td>58.2</td>
<td>30.0</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>44.3</td>
<td>38.0</td>
<td>17.7</td>
</tr>
<tr>
<td>dThd($\alpha - 5$)hdThd 2</td>
<td>56.2</td>
<td>31.0</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>41.3</td>
<td>39.0</td>
<td>19.7</td>
</tr>
<tr>
<td>dThd(6-4)dPyo 1</td>
<td>53.2</td>
<td>35.0</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>32.3</td>
<td>46.0</td>
<td>21.7</td>
</tr>
<tr>
<td>dThd(6-4)dPyo 2</td>
<td>54.2</td>
<td>30.0</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>57.2</td>
<td>34.0</td>
<td>8.8</td>
</tr>
</tbody>
</table>

(1) Calculated according to $g^+ = 13.75 - J(4'5') + J(4'5'\prime)/10.05$

(2) Calculated according to $t(\phi) = J(4'5'\prime)-1.5/10$
that the substrate be able to assume the \textit{anti} conformation, and that perhaps a \textit{syn} to \textit{anti} conformational isomerism is a natural part of the various catalytic processes.

A similar phenomenon has been observed for 8-bromoadenosine diphosphate which in solution assumes a preferentially \textit{syn} conformation, but is \textit{anti} when bound at the active site of liver alcohol dehydrogenase (155).

5.2. Conformational analysis of the new thymidine degradation products.

5.2.1. dThd(α-6)hdThd.

For both isomers we note a preference for the S (2'-endo) ring conformation for both rings, being more marked for the furanose attached to the saturated base. For the unsaturated nucleoside residue, we have a preferred \textit{anti} conformation as might be expected for a pyrimidine having no bulky C6 substituent. This is confirmed by a significant nuclear Overhauser effect between the H6 proton and the osidic hydrogens at positions 1', 2' and 3' (cf Section 1.4.2.2). The reverse is observed for the saturated residue since we now have a bulky thymidyl residue at C6 introducing a preference for the \textit{syn} conformation indicated by a downfield shift of the H2' signal of 0.23 ppm (0.3 ppm). We note also a marked reduction in the g\textsuperscript{+} conformation for the furanose exocyclic hydroxymethyl group due to the proximity of the C2 carbonyl group with a corresponding increase in the contributions from the t(ϕ) and particularly the g\textsuperscript{-} conformer. The degree of puckering $\tau\text{\textsubscript{m}}$ also varies considerably between the two residues. For the unsaturated moiety, the degree of puckering of 38.3° resembles that observed for thymidine (38.2°) (156). That for the saturated nucleoside residue is markedly less at 27.5°, interestingly the same trend was observed for 5,6-dihydrothymidine which showed a distinct reduction in the degree of puckering (31.6°) (157) compared to thymidine.

5.2.2. dThd(α-α)dThd.

The two nucleoside residues are in this case equivalent both chemically and magnetically with a plane of symmetry passing through the centre of the ethyl group linking the pyrimidine rings. Again the degree of puckering is of
the order of that of thymidine at 38.0°. The sugar conformation is preferen-
tially S with a value similar to that observed for the majority of the unsatu-
rated thymidyl residues in the new thymidine adducts. We observe no downfield
shift of the H2' signal indicating a preference for the anti conformation,
indeed the H2" signal is observed at lower field no doubt due to the greater
proximity of the hydroxyl group at the 3' position. There is also a preference
for the g⁺ rotamer although less than that observed for the thymidyl residue
of dThd(α-6)hdThd. Free rotation about the centre of the ethyl group allows
the nucleoside residues to assume any conformation with a minimum of steric
hindrance from the adjacent nucleoside, and behave almost as independent
thymidine residues.

5.2.3. dThd(3-5)hdThd.

The conformational parameters of the two isomers of the dThd(3-5)hdThd
adducts are very similar. For the unsaturated residue the value of τm is
slightly superior to that observed for the previous unsaturated thymidyl resi-
dues and the preference for the S (2'-endo) conformation is less marked. For
the first isomer there is only a slight downfield shift of the H2' signal
(0.07 ppm) for the saturated nucleoside residue suggesting an essentially anti
conformation. For the second isomer the downfield shift is somewhat greater at
0.12 ppm but is not large enough to justify the assignment of a significantly
syn conformation. For both isomers, the exocyclic groups of each furanose ring
shows a preference for the g⁺ rotamer, especially for the unsaturated thymidyl
residues. The decrease in the g⁺ population for the 5,6-dihydrothymidyl moie-
ties may reflect an increase in the amount of syn contribution. It is notable
that the anomeric proton signals for the unsaturated residues are at lower
field than those of the corresponding saturated nucleoside. Generally, the
opposite is observed. This may be an indication of a XCN of the order of
between -45° to -90° bringing the carbonyl group at C2 in close proximity to
the anomeric proton, being effectively coplanar at XCN = -60°. Paradoxically,
however, we would expect a positive shift in the value of XCN to account for
the downfield shift in the H2' signal. A Dreiding model of these molecules
shows that the carbonyl groups on the thymidyl residue are too far from the
osidic protons, whatever the conformation, to account for such a downfield
shift in the anomeric proton signal. It is difficult to explain the simulta-
neous downfield shifts of both H2' and H1' purely on the basis of syn/anti
considerations.
5.2.4. dThd(α-3)dThd.

It is unfortunately not possible at the moment to assign a particular base moiety to a furanose residue with any certainty, in the absence of nuclear Overhauser effect studies. The anomeric protons are isochronic but a NOE might be observed between the H2' and H3' protons and the alkenic H6 proton on one of the base residues. Both rings show normal degrees of puckering. We note however that the contributions of the 3'-endo conformation approach 50% in both cases. For one of the bases, the percentage of the t(ϕ) rotamer of the exocyclic group is as important as that of the normally preferred g+ conformation. It is apparent from the chemical shifts of the H2' signals that the nucleoside residues are both preferentially anti. If the assignment of the N3-methylene linkage is correct, it is evident from the model that in the anti conformation, the H6 and exocyclic hydroxymethyl groups will be in close proximity to the carbonyl groups of the N-bonded thymidyl residue. The result is an observed downfield shift of the corresponding H6 NMR signal at 8.053 ppm and a destabilisation of the g+ rotamer.

5.2.5. dThd(5'-6)hdThd.

The two isomers obtained for the dThd(5'-6)hdThd adducts differ greatly in conformation. For the first isomer, the unsaturated nucleoside residue comprises a furanose which has an average τm value at 39.5° as does that attached to the saturated base (36.2°). Both furanose units appear to show the generally preferred 2'-endo conformation. The downfield chemical shift of the H2' signal of the saturated nucleoside is lower than might be expected considering the size of the substituent at C6, although it is large enough to demonstrate a significant contribution from the syn conformation. The saturated nucleoside H2' signal also shows a downfield shift indicating that neither nucleoside residue shows any strong preference for the anti conformation. This notable syn contribution for the unsaturated nucleoside may be explained by the bulky group which the C6 carbon in the anti conformation sees attached to the 5' position of the corresponding sugar and which would tend to induce a movement away from the centre of the anti range. We might also note a significant downfield shift of the H6 NMR signal (8.002 ppm) suggesting that this proton is brought into close proximity to the ether furanose ring oxygen and perhaps also the 5'-OH function consistent with a XCN of approximately
0°, the centre of the anti range generally lying at -30°. The \( \text{g}^+ \) and \( t(\phi) \) rotamers of the only intact exocyclic group are equally important owing to the syn contribution.

According to the \(^1\)H NMR data, the second isomer shows a completely different conformation. The unsaturated nucleoside residue contains a furanose which is more than 80\% 2'-endo, the saturated residue showing a blend richer in the 3'-endo conformer, perhaps somewhat higher than generally observed for saturated thymidine residues. For the unsaturated nucleoside, the degree of pucker is quite low compared to that observed for thymidine at only 28°. We note a significant downfield shift for the H2' signal for this moiety of 0.18 ppm indicating a syn conformer contribution although we might assume a primarily anti conformation. The saturated residue however shows all the signs of an almost pure syn conformation. The H2' downfield shift is very large at 0.6 ppm and we detect also a significant upfield shift for the H1' signal (5.751 ppm) again indicative of the syn conformation. The exocyclic group now shows a distinct preference for the \( t(\phi) \) rotamer consistent with the destabilisation of the \( \text{g}^+ \) rotamer due to the proximity of the carbonyl group.

5.2.6. The spore photoproducts - dThd(α-5)tdThd.

Both isomers show similar \(^1\)H NMR characteristics. They both show a preference for the S conformation for both sugar moieties. The unsaturated base of each isomer adopts an essentially anti orientation relative to the sugar, however for the saturated residue we note a slight downfield shift of the H2' signal relative to the H2'' signal of 0.15 ppm indicating a slight shift towards the syn conformation. The degree of puckering is not unexpected for any of osidic residues. The exocyclic hydroxymethyl groups prefer the \( \text{g}^+ \) conformation.

5.3. Configurational and conformational analysis of the 5',6-cyclo-5,6-dihydropyrimidines.

5.3.1. 5',6-Cyclo-5,6-dihydrothymidine.

The assignment of a particular configuration to a particular isomer is hindered by the fact that the presence of three rings, one five-membered and two six-membered, presents the problem of a variety of possible conformations.
as a complicating factor. We are however helped in our analysis by the fact that the molecule is to a certain extent rigid in that the conformation of one ring is tightly linked to that of the others and that free rotation about single bonds is limited. In the following discussion we shall refer to each ring assigned as illustrated in Figure 5.4.

![Figure 5.4](image)

**Figure 5.4** : A perspective view of the three rings of the 5',6-cyclo-5,6-dihydropyrimidines.

We might first make some general comments about each of the rings. The base ring, A, generally assumes a twist-boat or half-boat conformation. The sugar ring, B, is almost completely rigid. The four ring carbons are planar, and in addition the oxygen atom is forced out of this plane by formation of the 5'-6 covalent bond. If the rings A and C lie above the plane of the sugar, the sugar ring oxygen lies below. Finally, it is the conformation or the newly formed ring which has the most profound effect on the detailed NMR features of a particular isomer. We should make some important comments about the stereochemistry of the N1 atom which is concyclic to rings A and C. It is known that, in unsaturated pyrimidines, the N1 lactam nitrogen is effectively
planar. Upon bond formation between C5' and C6, the newly formed ring will prefer the chair conformation, the other members of the ring being sp^3 hybridised, as is the C1' carbon atom. To suggest that this induces a pyramidal arrangement of the bond about N1 is speculative, however we might expect the most stable conformation to lie somewhere between the pyramidal ammonia and sp^2 planar type conformations. We shall therefore refer to chair, boat and twistboat conformations when discussing the conformations of ring C. Ring C may indeed assume all these conformations given free inversion at N1. It should be noted that conversion between chair and boat conformations of ring C also induces inversion of the half-boat conformation of ring A and would therefore be expected to greatly increase the associated energy barrier.

We shall discuss the configuration assignment of each isomer separately. Using a Dreiding model of the various possible configurations of 5',6-cyclo-5,6-dihydrothymidine, the experimental coupling constants (see Table I.3) were compared to those calculated according to Karplus (161) relating the J values to the dihedral angles between adjacent protons on an ethane-like residue. The likely configurations of the various isomers are given in Table V.4.

<table>
<thead>
<tr>
<th>ISOMER</th>
<th>AT C5'</th>
<th>AT C6</th>
<th>AT C5</th>
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<tr>
<td>I</td>
<td>S</td>
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<td>II</td>
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<td>S</td>
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<tr>
<td>VI</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

Isomer I is assigned the 5'S6S5R configuration. The values of J(5'6) and J(56) indicate strong coupling which may best be accounted for by a configuration where these three protons are all axial on a chair conformation. The 5'S6S5R configuration has protons which are axax on the ring C chair and flagpole on the ring A half-boat. This configuration also gives the expected value for J(4'5') of approximately 5 Hz typical of a gauche conformation, in this case equatorial and axial respectively on the ring C chair.
Isomer II may be obtained by leaving Isomer I in water for approximately 48 hours whereupon an almost 50:50 mixture of these isomers is obtained. This interconversion is undoubtedly due to a keto-enol tautomerism induced epimerisation at C5. We may therefore propose the 5'S6S5S configuration for Isomer II. Indeed this isomer shows a strong coupling between H5' and H6 almost identical to that observed for Isomer I, and the expected smaller J(56) value of 4 Hz, these protons now being equatorial and axial respectively.

The next two isomers have the particularity of not only having close to zero J(1'2') and J(3'4') coupling constants, but also the value of J(4'5') also lies between 0-1.5 Hz indicating an almost orthogonal dihedral angle, the H4' signal now appearing as a broad singlet through very weak coupling to H3', H5' and H2'' (long range). Whatever the configurations at C5', C6 and C5, we cannot account for a 90° dihedral angle between H4' and H5' when ring C assumes a chair conformation. For the two isomers ring C must assume a chair conformation and the configuration at C5' must be R in order for H4' and H5' to be orthogonal rather than eclipsed. The two possibilities for these products are 5'R6R and 5'R6S. Once we have assigned a particular configuration to each isomer the assignment of the configuration at C5 is straightforward since in each case C6 is axial and therefore according to the value of J(56) we can assign H5 as axial or equatorial.

In both cases, the configuration and conformation accounts for the weak coupling between H4' and H5', these protons being bowsprit and flagpole respectively. On a veritable cyclohexane-type boat conformer, these hydrogens would be gauche (120°), however owing to the presence of the ring oxygen which forms part of the severely puckered five-membered osidic ring and the fact that at the ring nitrogen the arrangement is not a true tetrahedron, this angle is reduced to approximately 100°. We are unable to assign a particular configuration to Isomers III and IV on the basis of the observed coupling constants, however the analysis of the 2'-deoxyuridine cyclic derivatives provides important information and allows us to make probable assignments. This will be discussed below in the section describing the analysis of these products.

The above is also true of the assignments of Isomers V and VI and we shall therefore introduce the data found for the 2'-deoxyuridine derivatives before discussing their configurations.
5.3.2. 5′,6-cyclo-5,6-dihydro-2′-deoxyuridine.

As expected for a compound containing two asymmetric carbon atoms, four isomers are possible and have all been isolated during the present study. The configurational analysis is facilitated compared with those isolated in the thymidine study having one asymmetric carbon less than the latter compounds. The structural analysis of each group of diastereoisomers is difficult when each group is considered separately, however a comparison of the two groups together allows a more confident assignment of the configuration and conformation in each case. It is interesting to note that some diastereoisomers of 5′,6-cyclo-5,6-dihydrothymidine show remarkably similar NMR characteristics to certain diastereoisomers of 5′,6-cyclo-5,6-dihydro-2′-deoxyuridine. The 1H NMR spectra for all four isomers of 5′,6-cyclo-5,6-dihydro-2′-deoxyuridine have been recorded at 400 MHz and the chemical shifts and coupling constants presented in Table III.3.

It is worth noting that in each case, H6 is always axial or flagpole since the very strong coupling, assigned as J(56) means that it is always \textit{trans} to a proton at C5. We can therefore eliminate all conformations where H6 finds itself bowsprit on the base ring twistboat.

For Isomer I, we may safely compare the configuration of this isomer with that of Isomer I of the cyclothymidines, where we note a very close resemblance between the values of J(4'5') and J(5'6). No other chair conformation and configuration can account for the observed coupling constants, we are therefore fairly certain of our attribution of 5'S6S.

Isomer II resembles none of the thymidine analogues in that it demonstrates a strong coupling between protons H4' and H5'. This can only be explained by a conformation where these protons are eclipsed. No ring C chair conformation is acceptable since these protons are always gauche. The conformation of ring C must be boat, and in addition, the configuration at C5' must be S. The two possible configurations which may account for the observed large coupling constant J(4'5') are 5'S6S or 5'S6R. Since we have attributed with confidence the former configuration to Isomer I, we must assign Isomer II as 5'T6R.

For Isomer III, we must also assign a boat conformation with the 5'R configuration this time in order to explain the very small coupling between protons H4' and H5'. Again, as in the case of the thymidine analogues, two
configurations are possible, that is 5'R6R and 5'R6S. The coupling constants for this isomer resemble those of Isomer IV of 5',6-cyclo-5,6-dihydrothymidine and we may tentatively assign the same configurations at these centres.

Finally, for Isomer IV, we note a close resemblance between the couplings J(4'5') and J(5'6) of this and Isomers V and VI of the cyclothymidine. We cannot make a definite assignment of the configuration of this isomer until we have resolved that of Isomer III.

Let us make certain comments concerning the possible configurational and conformational assignments for the cyclopyrimidine nucleosides, and see what information one group of isomers affords the analysis of the other.

- For 5',6-cyclo-5,6-dihydrothymidine, we have two isomers which are distinct and do not appear to undergo interconversion through epimerisation at C5, although we cannot rule out this possibility, and which must be 5'R on a ring C boat. We can account for the isolation of only two isomers in two ways. Firstly, that each isomer has a different configuration, i.e. 5'R6R and 5'R6S, and that each has its preferred configuration at C5. Alternatively, there might exist a common configuration at C5' and C6 and epimerisation at C5 may be very slow. This would mean that there remains one configuration to be isolated, in order to explain the isolation of only six of the possible eight isomers.

- For 5',6-cyclo-5,6-dihydro-2'-deoxyuridine we are certain to have all the possible isomers. We note two isomers where ring C is apparently chair, and two where the conformation is boat. The attributions appear quite clear for two isomers. For Isomer I, 5'S6S is the only possible configuration with the chair conformation which can account for the experimental coupling constants. Isomer II is necessarily 5'S with a ring C boat conformation in order for H4' and H5' to be eclipsed, and, in addition, must be 6R in order to distinguish it from Isomer I. For Isomer III, the conformation at C5' must be R and ring C must be boat. There appears now to be a certain amount of ambiguity since both 5'R6R and 5'R6S may assume a boat conformation and therefore we would apparently not be able to distinguish between these two possibilities and make unambiguous assignments for Isomers III and IV. One solution to the structural assignment is however more likely than the other.
If, for Isomer III, the configuration were 5'R6R, protons H5' and H6 would find themselves both flagpole on the ring C boat, and according to the model would have a dihedral angle of very close to 180°. The observed coupling between these two protons is lower than that expected at 8.1 Hz, but higher than that normally expected for a gauche spatial relationship. If, however, Isomer III were 5'R6S, these protons would be gauche but with very little distortion of the molecule we approach an eclipsed situation which would be in keeping with the observed coupling constant. Isomer IV would then have the 5'R6R configuration. Isomers V and VI of the of the thymidine product have coupling constants very similar to the latter isomer of the 2'-deoxyuridine analogue and therefore we might assign them the same configurations at C5' and C6.

In view of the strong similarity between the coupling constants of ring C protons for Isomer III of 5',6-cyclo-5,6-dihydro-2'-deoxyuridine and Isomer IV of 5',6-cyclo-5,6-dihydrothymidine, we might suggest the 5'R6S configuration for the latter. Isomer IV shows a weak coupling between H5 and H6 and hence H5 must be equatorial. This fixes the configuration at C5 as R.

If this assignment is correct, and hence that of Isomer IV of the 2'-deoxyuridine derivative, then Isomer III of 5',6-cyclo-5,6-dihydrothymidine must be the C5 epimer of Isomer IV since 5'R6R , the other configuration yielding a ring C boat and a small J(4'5') value, is assigned to a chair conformation and Isomers V and VI. For the latter isomers, we can go further and assign the C5 configurations for each. In the ring C chair conformation, H6 is flagpole on the ring A boat and hence we would expect one isomer to show a strong coupling between H6 and H5 (flagpole-flagpole) and the other to show a weaker coupling typical of a gauche pair (flagpole-bowsprit). Indeed for Isomer V this coupling is 11.7 Hz giving us the 5'R6R5S configuration and allows us to assign Isomer VI as 5'R6R5R, which coincidentally shows a weaker J(56) value. Consideration of the Dreiding model of this structure indicates a 5'6 dihedral angle of approximately 80° and explains the small coupling constants observed between these protons for each isomer.

It is apparent then that there remain two more isomers of 5',6-cyclo-5,6-dihydrothymidine to be isolated having the 5'S6R configuration.
EXPERIMENTAL

A. MATERIALS.

A.1 Commercially obtained starting materials.

Nucleosides were obtained from Pharma Waldorf and used without further purification. Deuterated solvents for NMR, $^{14}$CH$_3$-thymidine, paraformaldehyde-D$_2$, 18-oxygen and deuterium gas were all obtained from the Service des Molécules Marquées, Saclay, Paris.

A.2 Synthetic starting materials.

A.2.1. 5-bromo-6-hydroxy-5,6-dihydrothymidine.

Thymidine (3 g) was added to bidistilled water (30 ml) and the obtained suspension stirred in an icebath for 20 minutes. An excess of bromine (2.2 g) was added dropwise and the mixture stirred for 1 hour at 0 °C. The pH of the reaction mixture was controlled throughout the reaction period and kept close to neutrality by additions of sodium acetate. The excess bromine was then removed by passage of air for 10 minutes and the last traces removed under reduced pressure on a rotary evaporator without heating and without excessive concentration of the solution. The mixture was purified on a preparative reversed phase HPLC column whereby unreacted thymidine and the trans (+) and trans (-) diastereoisomers are efficiently separated. The fractions containing the two isomers of thymidine bromohydrin are evaporated to a small volume (50 ml) on a rotary evaporator and subsequently either frozen and stored as such, or lyophilised and stored at -20 °C. Yield 65 % (trans (+) (5R6R) : trans (5S6S) = 2:1).

A.2.2. Thymidine-CD$_3$.

A.2.2.1. 5-hydroxymethyl-2'-deoxyuridine-CD$_2$OH.

A solution comprised of 2'-deoxyuridine (3.42 g, 0.015 moles), paraformaldehyde-D$_2$ (6.0 g, 0.18 moles), NaOD (1N in D$_2$O, 15 ml) and D$_2$O (99.8%, 15 ml) was boiled under reflux for 8 hours, and the reaction mixture controlled by silicagel t.l.c. using Solvent II (see Section B.I.2.). The reaction was halted by cooling in an icebath, addition of bidistilled water (50 ml) and neutralisation with Dowex H cation exchange resin. The residue was filtered and evaporated to dryness. By silicagel analytical HPLC using Solvent II, the yield was estimated at 90%. The product gave a $^1$H NMR spectrum in D$_2$O identical to that of the authentic non-deuterated compound except that the two proton singlet in the region of 4.3 ppm was absent. Although not required for the subsequent reduction to thymidine, the deuterated 5-hydroxymethyl-2'-deoxyuridine may be purified by silicagel HPLC using Solvent II, the k' values for 5HMdUrd and dUrd being 4.7 and 2.4 respectively.
A.2.2. Thymidine-CD₃.

The reaction mixture obtained above (5.9 g) was freeze-dried and used without further purification. An approximately 50% solution of acetic acid in D₂O was prepared by adding acetic anhydride (80 ml) to deuterium oxide (99.8% isotopic purity, 100 ml) and leaving to stand overnight. This solution was added to the crude 5-hydroxymethyl-2'-deoxyuridine mixture and the solution obtained transferred to a 1 litre round-bottomed flask. Working in an efficient fume-cupboard, a rhodium/alumina catalyst (1.5 g) was added followed by deuterium gas and the flask stoppered and sealed with parafilm. The mixture was shaken mechanically for 9 hours. Every hour the mixture was controlled by analytical reversed phase HPLC using water-methanol (90:10 v/v) eluent. At 3 hourly intervals, further catalyst (1.5 g) and deuterium gas were added. The reaction mixture was then filtered using a millipore filter to remove the catalyst and the filtrate evaporated to dryness. Residual acetic acid was removed by evaporation from ethanol. Thymidine deuterated at the methyl group was purified by preparative reversed phase HPLC using water-methanol (90:10 v/v) eluent. The FAB mass spectrum gave a molecular weight of 245 amu. ¹H NMR showed a negligible methyl proton signal. Yield 1.63 g (45% based on the starting amount of 2'-deoxyuridine).


A.3.1. Thymidine 5'-aldehyde.

A.3.1.1. 5'-O-tritylthymidine.

Triphenylchloromethane (3.2 g) were added to thymidine (2 g) in anhydrous pyridine (15 ml). After ten days, the crystals of pyridine hydrochloride formed were removed by filtration and the filtrate evaporated to dryness under reduced pressure. The residue was taken up in a mixture of water (100 ml) and ethyl acetate (150 ml). The inferior water phase was removed and washed with ethyl acetate (2 x 50 ml). The ethyl acetate phase and washings were combined, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was taken up in toluene (200 ml) and the resulting solution evaporated slowly on a rotary evaporator. During the evaporation, the 5'-O-tritylthymidine precipitated and was harvested by filtration on a Buchner funnel. Yield 85%.

A.3.1.2. 3'-O-acetylthymidine.

5'-O-tritylthymidine (4 g) were dissolved in anhydrous pyridine (50 ml) and acetic anhydride (4 ml) added dropwise. The flask was sealed and left to stand at room temperature overnight. The solution was then evaporated to dryness under reduced pressure and 80% acetic acid (50 ml) added. The resulting solution was heated under reflux for ten minutes, allowed to cool and poured slowly into iced water (300 ml). The triphenylcarbinol which precipitated was removed by filtration on a Buchner funnel and the filtrate evaporated to dryness. The residue was recrystallised from ethyl acetate yielding sharp white needles. Yield 80%, mp 160 °C.
A.3.1.3. Thymidine 5'-aldehyde.

3'-O-acetylthymidine (700 mg) was dissolved in dry DMSO (15 ml) and phosphorus pentoxide (180 mg) and N,N-dicyclohexylcarbodiimide (1.31 g) added sequentially. The flask was sealed and the mixture left with stirring at ambient temperature overnight. The reaction was stopped by addition of water-methanol 50:50 (30 ml) and the mixture left to stand for 10 minutes. The precipitate of N,N-dicyclohexylurea was removed by filtration at the pump and the filtrate evaporated to dryness under vacuum. To the residue was added a saturated solution of ammonia in methanol (15 ml). After 2 hours, the solution was evaporated to dryness and the residue purified by reversed phase HPLC using water-methanol (95:5 v/v) eluent. The product eluted at k' 3.22 (thymidine k' 5.44) was collected and evaporated to dryness. Yield 53 %.

$^1$H NMR (D$_2$O, TSP) 1.90 (s, CH$_3$), 2.38 (dd, H$_2'$H$_2''$), 3.94 (dd, H$_4'$), 4.59 (m, H$_3'$), 5.15 (d, H$_5'$), 6.36 (t, H$_1'$), 7.76 (s, H$_6$).

A.3.2. l-(2-deoxy-$\alpha$-D-threo-pentafuranosyl)thymine.

Thymidine 5'-aldehyde (500 mg) was dissolved in pyridine (5 ml) and heated with stirring under a reflux condenser for 1 hour at 70 °C. The mixture was evaporated to dryness under vacuum and the residue taken up in methanol (15). Sodium borohydride (200 mg) was added and the mixture allowed to stand for 30 minutes. The reaction was stopped by addition of acetic acid (1 ml) and the mixture left to stand for 10 minutes before being evaporated to dryness. The l-(2-deoxy-$\alpha$-D-threo-pentafuranosyl)thymine (30 %) (k' 4.8) was separated from the l-(2-deoxy-$\beta$-D-erythro pentafuranosyl)thymine (70 %) (k' 8.3) by reversed phase semi-preparative HPLC using water-methanol (95:5 v/v) eluent.

B. ANALYTICAL METHODS.

B.1. Chromatographic techniques.

B.1.1. Analytical HPLC.

The apparatus used for analytical or semi-preparative HPLC separations was a composite Waters Associates apparatus comprised of:

- a M6000 pump
- a Rheodyne injector with a 2 ml loop (RP) or a 600 ul loop (silicagel)
- a refractometric detector

The columns used were generally home made of 1/4" or 3/8" internal diameter and using the following phases:

- Partisil silicagel 10 um
- Grafted C18 silicagel 10 um (Macherey-Nagel)

In one case an N-capped Waters Ass. ODS3 reversed phase column was used.
B.1.2. Preparative HPLC.

Large scale injections (1-3 g) were carried out on a Waters Associates LC/500 preparative HPLC apparatus equipped with a reversed phase Prep Pak 500/C18 column (5.7 cm diameter, 30 cm long) and a refractive index detector.

B.2. Thin layer chromatography.

B.2.1. Materials.

Two dimensional thin layer silicagel chromatography analyses were carried out using the following materials:

- MERCK F60/254/plastic (silicagel phase containing a 254 nm fluorescent indicator mounted on plastic sheets (20 x 20 x 0.02 cm).
- Solvent I: Chloroform-methanol-water (4:2:1 v/v/v) (100 ml lower phase + 5 ml methanol).
- Solvent II: Ethyl acetate-propan-2-ol-water (75:16:9 v/v/v).

B.2.2. Detection of products.

Base unsaturation - irradiation of the t.l.c. plate with 254 nm light after migration revealed, owing to the presence of the fluorescent marker, the spots due to those products absorbing at that wavelength.

Osidic moiety - the 2-deoxyribose residue of nucleosidic products was revealed using the Buchanan reaction (158) involving spraying and subsequent heating (100 °C for 10 min.) of the plate with a cysteine reagent comprising 0.4% cysteine in 3N H2SO4 (aq). 2'-deoxyribonucleosides are revealed as spots ranging from black to violet to pink depending on the product and plate load.

Radioactive products - radioactive products in quantitative studies were located by autoradiography using KODAK definix plates, 16 hours exposure allowing the detection of approximately 0.01 μCi.

B.3. Spectroscopic techniques.

B.3.1. Nuclear magnetic resonance spectroscopy.

The 1H and 13C NMR spectra were recorded in deuterium oxide (D2O 99.95%), acetone-d6 (CD3 COCD3 99.7%) and dimethyl sulfoxide-d6 (DMSO-d6 99.8%) obtained from the Service des Molecules Marquées, Saclay, France.

The following spectrometers were used:
- Bruker AM400 equipped with an Aspect 3000 computer in FT mode.
- Bruker WM250 equipped with an Aspect 2000 computer in FT mode.
- Bruker AM200 equipped with an Aspect 3000 computer in FT mode.
B.3.2. Mass spectrometry.

B.3.2.1. Fast atom bombardment (FAB) mass spectrometry.

Mass spectrometry measurements were made using a GEC-AEI MS-50 apparatus using the Fast Atom Bombardment technique (2), the sample presented as a glycerol mull under a bombardment of 5-8keV argon atoms. The mass spectral data were treated with an IBM 360-50 computer.

B.3.2.2. Electron impact (E.I.) mass spectrometry.

The mass spectra were recorded using a GEC-AEI MS-50 apparatus. The sample was introduced directly into the ion source at a temperature such that a stable vapourisation was obtained throughout the recording of the spectrum (approx. 150 °C). The data were treated on an IBM 360-50 computer.

B.3.2.3. Desorption chemical ionisation (DCI) mass spectrometry.

The spectra were recorded using a Nermag R1010 apparatus equipped with a quadrupole ion separator in the positive mode. The source temperature was 100 °C and the source medium comprised an ammonia/isobutane atmosphere of 1 torr. The sample was placed onto a filament which was rapidly heated to approximately 700 °C (filament current increase from 20-400 mA at a rate of 10 mA/sec).

B.3.3. UV and visible absorption spectroscopy.

UV absorption spectra were recorded in water on a Beckman DU-8B and a Beckman UV 5230 spectrophotometer.

C. GAMMA IRRADIATION OF THYMIDINE IN FROZEN AQUEOUS SOLUTION.

C.1.1. Irradiation conditions and isolation procedure for standard experiment.

Thymidine (10.89 g) was dissolved in bidistilled water (pH 6.5, 150 ml, 0.3 M) in a 250 ml cylindrical flask with gentle warming and the resulting solution deaerated by passage of nitrogen gas for 10 minutes. The flask was then sealed and the solution rapidly frozen by immersion in liquid nitrogen. The sample was packed in dry ice and irradiated at 196 K for 384 hours at a dose rate of 1.12 Mrad/hr (total dose 4.30 MGy). The sample was then allowed to anneal slowly to room temperature and evaporated to dryness on a rotary evaporator. Undegraded thymidine was removed by successive precipitations andfiltrations from hot ethanol down to a volume of 20 ml. The final filtrate was evaporated to dryness and the residue taken up into 50 ml of water-methanol (90:10 v/v). The resulting solution was injected on to a preparative reversed phase HPLC column. The sample was eluted with 3 litres of water-methanol (90:10 v/v) resulting in the elution of the remaining thymidine, followed by 1 litre of water-methanol (80:20 v/v), and the column finally washed with 1 litre of methanol. The fractions obtained were evaporated to dryness, analysed by thin layer silicagel chromatography, and rechromatographed using analytical HPLC techniques.
C.1.2. PRODUCT ISOLATION

C.1.2.1. Preparative HPLC fractionation.

The final filtrate from the successive precipitations of thymidine was evaporated to dryness leaving a solid amorphous residue (1.1 g). This mixture was taken up in water-methanol (90/10 v/v, 50 ml), the resulting solution filtered, and injected on to a Waters Assoc. LC500 preparative HPLC apparatus equipped with a C18 reversed phase column using water-methanol (90/10 v/v) mobile phase at a flow-rate of 100 ml/min. The eluent was separated into five fractions, the last corresponding to the methanol column washings (see Appendix A.1).

C.1.2.2. Resolution of the first preparative HPLC fraction.

Preparative HPLC fraction A.1.1 (k' 0.6) was evaporated to dryness and rechromatographed on a semi-preparative reversed phase HPLC apparatus using the following conditions.

Column : 3/8" C18
Eluent : water
Flow rate : 3 ml/min
Detector : refractometer

The chromatogram (Appendix A.2) gave three peaks (peaks A.2.1-A.2.3) which were shown to contain the following products:

Peak A.2.1 (k' 1.14)

(5S6S) 5,6-dihydroxy-5,6-dihydrothymidine.
2-deoxy-D-ribo-1,4-lactone

Peak A.2.2 (k' 2.28)

(5R6R) 5,6-dihydroxy-5,6-dihydrothymidine.

Peak A.2.3 (k' 3.29)

5'R6R5S) 5',6-cyclo-5,6-dihydrothymidine (Isomer V).
(5R) 5-hydroxy-5,6-dihydrothymidine.
(5R5S) 5,6-dihydroxy-5,6-dihydrothymidine.
(5S6R) 5,6-dihydroxy-5,6-dihydrothymidine.

(5S6S) 5,6-dihydroxy-5,6-dihydrothymidine.

Reinjection of peak A.2.1 onto a silicagel HPLC column using Solvent II eluent gave two peaks, the first of which ( k' 1.80) was collected and evaporated to dryness yielding 4 mg of an amorphous white solid. The structure was assigned as that of (5S6S) 5,6-dihydro-5,6-dihydrothymidine by comparison of the $^1$H NMR data with those published in the literature (56).
2-deoxy-D-ribono-1,4-lactone

The second peak (k' 4.36) of the silicagel HPLC injection of peak A.2.1 was collected and evaporated to dryness yielding 5 mg of an amorphous white solid which was shown by $^1$H NMR to be 2-deoxy-ribono-1,4-lactone (159).

(5R6R) 5,6-dihydroxy-5,6-dihydrothymidine.

Evaporation of peak A.2.2 to dryness yielded 3 mg of an amorphous off-white solid and was shown by two-dimensional silicagel t.l.c. to consist of one nucleosidic product. $^1$H NMR in D$_2$O showed it to be (5R6R) 5,6-dihydroxy-5,6-dihydrothymidine (56).

(5R6R6S) 5',6-cyclo-5,6-dihydrothymidine (Isomer V).

The residue obtained on evaporation of peak A.2.3 to dryness (12 mg) was reinjected onto a silicagel column using Solvent II eluent. The resulting chromatogram showed the presence of three major peaks. The first of these (k' 1.47) was collected and evaporated to dryness whereupon 5 mg of an amorphous white solid was obtained. The structure was assigned as that of (5R6R6S) 5',6-cyclo-5,6-dihydrothymidine on the basis of its $^1$H NMR spectrum run in D$_2$O.

$^1$H NMR spectrum : see Table I.3

EI mass spectrum :
m/z (rel. abundance) 242 (5.7 M); 183 (2.5); 181 (3.5); 127 B ;
98 (7.1); 84 (43.3); 73 (12.9); 69 (5.7).

(5R) 5-hydroxy-5,6-dihydrothymidine.

The second peak from the silicagel chromatography of peak A.2.3 (k' 2.17) was evaporated to dryness to give 3 mg of an amorphous white solid. The structure assignment of (5R) 5-hydroxy-5,6-dihydrothymidine was made by comparison of its $^1$H NMR features with those reported in the literature (57).

(5R6S) and (5S6R) 5,6-dihydroxy-5,6-dihydrothymidine.

The third peak from the reinjection of peak A.2.3 (k' 3.23) was evaporated to dryness yielding 3 mg of an amorphous white solid which was shown by $^1$H NMR in D$_2$O to consist of a mixture of the cis thymidine glycols (56).

C.1.2.3. Resolution of the second preparative HPLC fraction.

The second preparative HPLC fraction (A.1.2, k' 1.81) was evaporated to dryness to yield 320 mg of an off-white solid.
Thymine.

The residue was taken up in 10 ml of bidistilled water with warming. On leaving the resulting solution to stand in an ice-bath for two hours, a white precipitate was obtained which was removed by filtration. The white powder obtained (70 mg) co-chromatographed with an authentic sample of thymine using Solvents I and II and gave a negative Buchanan test (158) with cysteine/H$_2$SO$_4$. The product was confirmed as being thymine by $^1$H NMR in D$_2$O.

The filtrate obtained above was rechromatographed using reversed phase HPLC using water as the mobile phase. The eluent was divided into five fractions (see Appendix A.3) which were shown to contain the following products:

Fraction A.3.1 (k' 4.71-5.28).
5,6-dihydrothymine.
(5'R6S5S) 5',6-cyclo-5,6-dihydrothymidine (Isomer III).
Trans (-)(5S6S) 6-hydroxy-5,6-dihydrothymidine.
(5S) 5-hydroxy-5,6-dihydrothymidine.

Fraction A.3.2 (k' 5.28-6.79).
Thymine.
Thymidine.
(5'R6S5R) 5',6-cyclo-5,6-dihydrothymidine (Isomer IV).
Cis (+)(5S6R) 6-hydroxy-5,6-dihydrothymidine.
Trans (-)(5S6S) 6-hydroxy-5,6-dihydrothymidine.

Fraction A.3.3 (k' 6.79-8.14).
(5'S6S5S) 5',6-cyclo-5,6-dihydrothymidine (Isomer II).
Trans (+)(5R6R) 6-hydroxy-5,6-dihydrothymidine.
Cis (-)(5R6S) 6-hydroxy-5,6-dihydrothymidine.
5-hydroxymethyl-2'-deoxyuridine.

Fraction A.3.4 (k' 8.14-11.28).
(5'S6S5R) 5',6-cyclo-5,6-dihydrothymidine (Isomer I).
(5S)(-) 5,6-dihydrothymidine.
5-hydroxymethyl-2'-deoxyuridine.
(5'R6R5R) 5',6-cyclo-5,6-dihydrothymidine (Isomer VI).
Cis/syn (-) cyclobutidithymidine.

Fraction A.3.5 (k' 11.28-16.57).
(5R)(+) 5,6-dihydrothymidine.
1-(2'-deoxy-α-D-threo pentofuranosyl)thymine.
trans/syn (-) cyclobutidithymidine.
Cis/syn (meso) cyclobutidithymidine.
Products of Fraction A.3.1.

5,6-dihydrothymine.

Reinjection of fraction A.3.1 on to silica gel using Solvent II eluent yielded four major peaks (Appendix A.4), the first of which (k' 1.58) was collected and evaporated to dryness. The white powder obtained (15 mg) gave no cysteine/H₂SO₄ test and showed no absorbance at 254 nm. The structure was confirmed by comparison of the ¹H NMR spectrum recorded in D₂O with that recorded for a commercially obtained sample of 5,6-dihydrothymine (Sigma).

(5'R6S5S) 5',6-cyclo-5,6-dihydrothymidine (Isomer III).

The second major peak (k' 2.42) from fraction A.3.1 was evaporated to dryness and yielded 4 mg of an amorphous white solid. The structure was assigned as (5'R6S5S) 5',6-cyclo-5,6-dihydrothymidine on the basis of ¹H NMR and EI mass spectrometric analysis.

¹H NMR: see Table I.3

EI mass spectrum:

m/z (rel. abundance) 242 (1.4 M); 181 (3.8); 127 (B); 98 (6.2); 84 (35.7); 73 (12.4); 69 (4.8).

Trans(-) 6-hydroxy-5,6-dihydrothymidine.

The third peak (k' 4.91) was evaporated to dryness whereupon 4 mg (total yield 10 mg, see below) of an amorphous white solid were obtained and which gave ¹H NMR data identical to those reported in the literature for trans (-) 6-hydroxy-5,6-dihydrothymidine (58).

(5S) 5-hydroxy-5,6-dihydrothymidine.

The fourth peak from the reinjection of fraction A.3.1 (k' 8.41) was collected and evaporated to dryness and the amorphous white solid obtained (1 mg) revealed by comparison of its ¹H NMR features with literature data to be (5S) 5-hydroxy-5,6-dihydrothymidine (57).

Products of Fraction A.3.2.

The second fraction obtained from semi-preparative reversed phase HPLC chromatography of A.1.2 was injected onto a 3/8" silicagel HPLC column using Solvent II eluent at a flow rate of 3 ml/min. The chromatogram showed the presence of five major peaks (see Appendix A.5). Peaks 1 (k' 1.33) and 2 (k' 2.17) correspond to residual thymine and thymidine from the spontaneous dehydration of the two isomers of 6-hydroxy-5,6-dihydrothymidine present respectively. The fifth peak (k' 4.91) was shown, like the third peak of the previous chromatogram, to be trans (-)(586S) 6-hydroxy-5,6-dihydrothymidine.
(5'R6S5R) 5',6-cyclo-5,6-dihydrothymidine (Isomer IV).

The third peak from the silicagel separation of fraction A.3.2 (k' 3.25) was evaporated to dryness yielding 2 mg of a white amorphous solid which was assigned, on the basis of $^1$H NMR and EI mass spectrometry, the structure of (5'R6S5S) 5',6-cyclo-5,6-dihydrothymidine.

$^1$H NMR : see Table I.3

EI mass spectrum :
m/z (rel. abundance) 242 (2.0 M ); 183 (2.1); 181 (3.8); 127 B ; 98 (8.1); 84 (38.1); 73 (13.8); 69 (7.6); 42 (18.1).

Cis(+)5S6R 6-hydroxy-5,6-dihydrothymidine.

Peak 4 (k' 3.50) was collected and evaporated to dryness giving 12 mg of an amorphous white solid, the $^1$H NMR characteristics of which were identical to those reported in the literature for cis (5S6R) 6-hydroxy-5,6-dihydrothymidine (58).

Products of fraction A.3.3.

Fraction A.3.3. was injected onto a semi-preparative silicagel column, the resulting chromatogram revealing the presence of four major peaks (Appendix A.6).

(5'S6S5S) 5',6-cyclo-5,6-dihydrothymidine (Isomer II).

The first peak from the silicagel chromatography of fraction A.3.3. (k' 1.92) was evaporated to dryness to give 15 mg of a white amorphous solid which, on the basis of $^1$H NMR and EI mass spectrometry analysis, was assigned the structure (5'S6S5S) 5',6-cyclo-5,6-dihydrothymidine.

$^1$H NMR spectrum : see Table I.3

EI mass spectrum :
m/z (rel. abundance) 242 (3.4 M ); 183 (6.3); 181 (6.7); 127 B ; 84 (28.6); 73 (8.6); 71 (7.1).

Trans(+)5R6R 6-hydroxy-5,6-dihydrothymidine.

The second peak from the separation of fraction A.3.3. on silicagel (k' 3.83) was evaporated to dryness wherupon 2 mg of a white amorphous solid was obtained. The $^1$H NMR spectrum in D$_2$O was identical to that reported in the literature for trans (5R6R) 6-hydroxy-5,6-dihydrothymidine (58).
Cis(-)(5R6S) 6-hydroxy-5,6-dihydrothymidine.

The third peak (k' 4.75) was collected and evaporated to dryness whereupon 0.5 mg of a white amorphous solid was obtained. The product was shown by $^1$H NMR to be cis (-)(5R6S) 6-hydroxy-5,6-dihydrothymidine (58).

5-hydroxymethyl-2'-deoxyuridine.

Evaporation to dryness of the fourth peak (k' 6.67) yielded 23 mg of a white crystalline solid. The product was characterised as 5-hydroxymethyl-2'-deoxyuridine on the basis of $^1$H NMR spectroscopy by comparison with literature data (59).

Products from fraction A.3.4.

Evaporation of fraction A.3.4 to dryness and subsequent reinjection on to a semi-preparative silicagel HPLG column using Solvent II eluent showed the mixture to be comprised of five products (Appendix A.7). The third peak was identified as 5-hydroxymethyl-2'-deoxyuridine of which the major portion was isolated in the previous fraction.

(5'S6S5R) 5',6-cyclo-5,6-dihydrothymidine (Isomer I).

The first peak from the silicagel purification of fraction A.3.4 (k' 1.83) was evaporated to dryness to yield an amorphous white solid (23 mg) the structure of which was assigned as (5'S6S5R) 5',6-cyclo-5,6-dihydrothymidine on the basis of $^1$H NMR and EI mass spectrometric analyses.

$^1$H NMR spectrum : see Table I.3

$^{13}$C NMR (D$_2$O, TMS) δ(ppm): 15.7 (q, CH$_3$), 41.4 (t, C2'), 42.2 (d, C5), 58.7 (d, C6), 70.3 (d, C3'), 70.9 (d, C5'), 86.0 (d, Cl'), 88.3 (d, C4'), 177.3 (s, C4).

EI mass spectrum : m/z (rel. abundance) 242 (4.5 M); 183 (7.6); 181 (4.2), 127 (8.6); 126 (3.8); 99 (2.5); 98 (7.1); 84 (25.9); 73 (9.5); 71 (6.2); 55 (4.8); 45 (2.4); 43 (2.8).

(5S) 5,6-dihydrothymidine.

The second peak (k' 4.00) was evaporated to dryness to yield 55 mg of an amorphous white solid giving identical $^1$H NMR data to those reported in the literature (61) for (5S) 5,6-dihydrothymidine.
Evaporation of the fourth peak from silicagel chromatography of fraction A.3.4 gave 3 mg of a white solid which was assigned the structure \( (5'R6R5R) \ 5',6\text{-cyclo-}5,6\text{-dihydrothymidine} \) based upon \(^1\)H NMR and EI mass spectrometries.

\(^1\)H NMR spectrum: see Table I.3

EI mass spectrum:
m/z (rel. abundance) 242 (4.3 M); 183 (2.4); 181 (3.4);
127 B; 98 (8.1); 84 (47.6); 73 (21.0);
55 (12.4); 45 (17.2); 43 (16.2).

Cis/anti(-) cyclobutidithymidine.

The last peak (\( k' \ 8.33 \)) was evaporated to dryness giving 2 mg of a white solid. The \(^1\)H NMR spectrum in D\(_2\)O revealed it to be the cis/anti(-) diastereoisomer of cyclobutidithymidine (61).

Products from fraction A.3.5.

Fraction A.3.5 was evaporated to dryness and injected onto a semi-preparative silicagel HPLC column using Solvent II as eluent (Appendix A.8). The mixture was shown to be comprised of four components.

\( (5R) \ 5,6\text{-dihydrothymidine} \).

Evaporation of the first peak (\( k_1 \ 2.67 \)) yielded 45 mg of a white amorphous solid which was identified as \( (5S) \ 5,6\text{-dihydrothymidine} \) by comparison of its \(^1\)H NMR data with those reported in the literature (61).

\( l-(2\text{-deoxy-} \alpha \text{-D-threopentofuranosyl})\text{thymine} \).

Evaporation of the second peak (\( k_R \ 3.33 \)) to dryness yielded 3 mg of a white amorphous solid which was characterised by \(^1\)H NMR in D\(_2\)O as \( l-(2\text{-deoxy-} \alpha \text{-D-threopentofuranosyl})\text{thymine} \).

\(^1\)H NMR (D\(_2\)O, TMS) \( \delta \) (ppm) 1.88 (s, CH\(_3\)), 2.41 (m, H2'),
2.53 (m, H2''), 2.80 (q, H5''),
3.88 (q, H5'), 4.44 (m, H4'),
4.61 (m, H4''), 6.26 (t, H1'),
7.53 (s, H6).

Trans/syn(-) cyclobutidithymidine.

The third peak (\( k' \ 5.00 \)) from the injection of fraction A.3.5 onto silicagel yielded, upon evaporation to dryness, 3 mg of a white solid yielding \(^1\)H NMR data identical to that reported in the literature for trans/syn (-) cyclobutidithymidine (61).
Trans/anti cyclobutidithymidine.

Peak A.9.2 was reinjected on a semi-preparative reversed phase column using Solvent II eluent, the chromatogram showing the presence of principally one product (k' 6.08). This fraction was collected and evaporated to dryness whereupon 8 mg of an amorphous white solid were obtained. The $^1$H NMR spectrum for this product matched that reported in the literature for trans/anti cyclobutidithymidine (61).

dThd($\alpha$-6)hdThd Isomer I.

Peak A.9.3 was reinjected on to a semi-preparative silicagel HPLC column using Solvent II eluent. The peak was shown to contain only one product (k' 8.08) which was collected and evaporated to dryness. A white amorphous solid (22 mg) was obtained and was assigned the structure 6-thymidyl-5,6-dihydrothymidine or dThd($\alpha$-6)hdThd based on $^1$H and $^{13}$C NMR and FAB mass spectrometric analyses.

$^1$H NMR : see Table I.2
$^{13}$C NMR : see Table I.4
FAB-MS : m/z 485 (MH$^+$); 507 (M + Na$^+$); 369 (MH$^+$ - dR); 243 (dThd + H$^+$); 127 (BH$^+$); 117 (dR$^+$).

UV (A$_{max}$, H$_2$O) 262 nm.

dThd($\alpha$-6)hdThd Isomer II.

Peak A.9.4 was rechromatographed using a semi-preparative silicagel HPLC column and was shown to contain two products. The first peak (k' 8.33) was collected and evaporated to dryness yielding a white amorphous solid (11 mg). The product was analysed by $^1$H and $^{13}$C NMR and FAB mass spectrometries and assigned the structure 6-thymidyl-5,6-dihydrothymidine, being isomeric with the product isolated from peak A.9.3.

$^1$H NMR : see Table I.2
$^{13}$C NMR : see Table I.4
FAB-MS : m/z 485 (MH$^+$); 507 (M + Na$^+$); 369 (MH$^+$ - dR); 243 (dThd + H$^+$); 127 (BH$^+$); 117 (dR$^+$);

UV (A$_{max}$, H$_2$O) 262 nm.

dThd($\alpha$-5)hdThd Isomer I.

The second peak (k' 11.3) from the silicagel chromatography of peak A.9.4 was evaporated to dryness yielding an amorphous white solid (3 mg). The structure assigned based on $^1$H and $^{13}$C NMR and FAB mass spectroscopic data was that of the spore photoproduct nucleoside dimer, dThd($\alpha$-5)hdThd.
The washings of the column after elution of the fourth peak of the semi-preparative reversed phase chromatography of the fourth and fifth preparative fractions were evaporated to dryness and rechromatographed on the same reversed phase column using water-methanol (90/10 v/v) eluent (Appendix A.10). From this mixture seven products have been identified.

dThd(α-5)hdThd Isomer II.

The first major peak from the injection of the methanol washings onto reversed phase (k' 6.89) was rechromatographed on a semi-preparative silicagel HPLC column whereupon one major peak was observed (k' 11.7). This peak was collected and evaporated to dryness. The white amorphous residue (3 mg) was shown by ¹H and ¹³C NMR and FAB mass spectrometries to be the second isomer of the spore photoproduct nucleoside dimer, dThd(α-5)hdThd.

FAB-MS: m/z 485 (MH⁺); 507 (M + Na⁺); 369 (MH⁺ - dR); 243 (dThd + H⁺); 127 (BH⁺); 117 (dR⁺);

dThd(3-5)hdThd Isomer I

The second peak (k' 8.89) was rechromatographed on silicagel using Solvent II eluent and gave a peak at k' 11.67 which on evaporation to dryness yielded 1 mg of an amorphous white solid. After analysis by ¹H NMR and FAB mass spectrometry, this product was assigned the structure dThd(3-5)hdThd.

FAB-MS: m/z 485 (MH⁺); 507 (M + Na⁺).

dThd(3-5)hdThd Isomer II.

The shoulder to the previous peak (k' 9.55) was injected in turn onto a semi-preparative HPLC column whereupon a peak having a k' value of 9.67 was collected and evaporated to dryness yielding an amorphous white solid (1 mg). ¹H NMR analysis revealed this product to be a structural isomer of that isolated from the previous peak, dThd(3-5)hdThd.
dThd(5'-6)hdThd Isomer I.

The reversed phase HPLC peak eluted at k' 11.44 was rechromatographed on a semi-preparative silica gel HPLC column using the Solvent II eluent. One major peak was obtained (k' 5.50) which on evaporation to dryness gave 1 mg of an amorphous white solid. Based upon the $^1$H NMR and FAB mass spectral data, the structure was assigned as dThd(5'-6)hdThd.

$^1$H NMR : see Table I.2
FAB-MS : m/z 485 (MH$^+$); 507 (M + Na$^+$).

\textit{dThd(\alpha-\alpha)dThd.}

The peak eluted at k' 12.89 from the semi-preparative reversed phase HPLC chromatography of the methanol washings was evaporated to dryness and purified on a silica gel HPLC column using Solvent II (k' 8.16). Upon evaporation, an amorphous white solid was obtained (5 mg) which was assigned the structure dThd(\alpha-\alpha)dThd based on $^1$H NMR and FAB mass spectrometric data.

$^1$H NMR : see Table I.2
FAB-MS : m/z 483 (MH$^+$); 505 (M + Na$^+$).

UV ($\lambda_{max}$, H$_2$O) 272 nm.

\textit{dThd(5'-6)hdThd Isomer II.}

The reversed-phase HPLC peak eluted at k' 15.34 was rechromatographed on a silica gel column giving one major peak (k' 6.78) which upon evaporation to dryness gave 0.5 mg of an amorphous white solid. $^1$H NMR showed it to be a diastereoisomer of the product isolated earlier and assigned the structure dThd(5'-6)hdThd.

$^1$H NMR : see Table I.2
FAB-MS : m/z 483 (MH$^+$); 505 (M + Na$^+$).

\textit{dThd(\alpha-3)dThd.}

The last peak from the chromatography of the methanol washings of the reinjection of the fourth and fifth preparative HPLC fractions was eluted at k' 21.67. Evaporation of this fraction to dryness and reinjection onto silica gel gave one major peak (k' 5.83). Evaporation of this peak to dryness yielded 2 mg of an amorphous white solid assigned the structure dThd(\alpha-3)dThd based on $^1$H NMR and mass spectrometric data.

$^1$H NMR : see Table I.2
FAB-MS : m/z 483 (MH$^+$); 505 (M + Na$^+$).
C.2. Gamma irradiation of thymidine in frozen deuterium oxide solution.

Thymidine (3.63 g) was dissolved in deuterium oxide (150 ml, 99.8%, 0.1 M) and degassed by passage of nitrogen gas for 10 minutes. The solution obtained was rapidly frozen by immersion in liquid nitrogen and the sample subsequently irradiated at 196 K for 60 hours at a dose rate of 1.12 Mrad/hr (total dose 6.72 x 10E5 Gy). The sample was allowed to thaw to room temperature and the following products isolated as described in Section C.1.2.

1. (5R) and (5S) 5,6-dihydrothymidine.
2. 5,6-dihydrothymine
3. (5R) 5-hydroxy-5,6-dihydrothymidine.
4. (5R6S) 6-hydroxy-5,6-dihydrothymidine.
5. (5'S6S5R) 5',6-cyclo-5,6-dihydrothymidine (Isomer I).
6. dThd(α-6)dhdThd (Isomers I and II).


Thymidine (1.21 g) was dissolved in bidistilled water (pH 6.5, 30 ml, 0.167 M) and the resulting solution degassed three times under vacuum. The sample was saturated with 18O2 (isotopic purity 99.8%) and rapidly frozen in liquid nitrogen. The sample was irradiated for 168 hours (total dose 1.88 MGy). The solution was then annealed, evaporated to dryness and the excess thymidine removed by successive precipitations from hot ethanol down to a final filtrate volume of 3 ml. The filtrate was evaporated to dryness, taken up into 4 ml of water-methanol (90:10 v/v) and the mixture fractionated on a 3/8" reversed phase column using the same solvent as eluent (8 x 500 ul). Three fractions were collected: before elution of the remaining thymidine, the thymidine peak, and methanol washings. The fraction eluted before thymidine was concentrated and rechromatographed on a reversed phase column using bidistilled water as the eluent followed by silicagel HPLC analysis of the fractions obtained. The following products were isolated as described in Section C.1.2.

1. 2-deoxy-D-ribono-4-lactone.
2. (5R6S) and (5S6R) 5,6-dihydroxy-5,6-dihydrothymidine.
3. (5R) 5-hydroxy-5,6-dihydrothymidine.
4. 5-hydroxymethyl-2'-deoxyuridine.


Thymidine-CD3 (1.21 g) was dissolved in bidistilled water (30 ml, 0.167 M) and the resulting solution degassed by passage of nitrogen for 10 minutes. The sample was rapidly frozen by immersion in liquid nitrogen and irradiated at 196 K for ten days (total dose 2.69 MGy). The sample was then slowly brought to room temperature and the major part of the remaining thymidine removed by successive precipitations from hot ethanol. The final filtrate was evaporated to dryness on a rotary evaporator and the residue taken up in 4 ml of water-methanol (90:10 v/v). This solution was fractionated on a 3/8" reversed phase analytical column (8 X 500 μl) using the same solvent as eluent and the fraction eluted after the
thymidine peak reinjected on to the same column using water-methanol (95:5 v/v) eluent. The peak eluting at k' 11.2 was shown by $^1$H NMR spectrometry to be deuterated form of the Isomer I of dThd(α-6)hdThd. The second peak at k' 18.2 was injected on to a semi-preparative silicagel HPLC column using Solvent II. The product eluting first (k' 8.33) was shown to be Isomer II of dThd(α-6)hdThd and that eluting second (k' 11.3) shown to be Isomer I of dThd(α-5)hdThd on the basis of $^1$H NMR data (Table I.2).

C.5. Quantitative analysis of the gamma radiolysis of thymidine in frozen aqueous solution.

(14CH$_3$)-thymidine was separated from its products of self-radiolysis by injection onto a reversed phase HPLC column (ODS3) using water-methanol (95:5 v/v) mobile phase. The labelled thymidine was then evaporated to dryness and to the residue a solution of cold thymidine (1 ml, 0.1 M) was added. Using this solution, the following samples were prepared.

1. Thymidine (0.01 M) + 2-iodoacetamide (0.01 M).
2. Thymidine (0.01 M).
3. Thymidine (0.01 M) + 2'-deoxyadenosine (0.01 M).
4. Thymidine (0.01 M) + 2'-deoxycytidine (0.01 M).
5. Thymidine (0.01 M) + 5-nitro-2-furoic acid (0.001 M).

The solutions were deaerated by passage of nitrogen for 10 minutes, rapidly frozen in liquid nitrogen and irradiated for 4 hours at 196 K (total dose 4.48 x 10$^4$ Gy). The samples were slowly annealed and two-dimensional thin layer silicagel chromatographs (20 x 20 cm) were run in Solvents I and II. The spots were located and identified by autoradiography and co-chromatography with authentic samples respectively. The silicagel was removed by scraping, the products eluted with water and counted by liquid scintillation (see Table I.5).


Trans (+) thymidine bromohydrin (815 mg) were dissolved in bidistilled water (30 ml, 0.08 M). A 5 ml aliquot was retained and kept frozen to serve as a control, the remainder of the solution obtained was degassed by passage of nitrogen gas for 10 minutes, rapidly frozen in liquid nitrogen and irradiated at 196 K for 80 hours (total dose 0.90 MGy). The sample was allowed to come to room temperature and injected on to a reversed phase preparative HPLC column using water-methanol (80:20 v/v) eluent. The dead volume peak was isolated from the rest of the eluent owing to the high concentration of hydrobromic acid in this fraction. The fraction eluted after this peak and before the undegraded thymidine bromohydrin was evaporated to dryness and the mixture resolved by analytical reversed phase HPLC using water as the eluent.

5-hydroxymethyl-2'-deoxyuridine: k'(RP) 7.95, yield 9 mg (59).

Thymidine: k'(RP) 21.4, yield 15 mg.

The samples should at no time be evaporated to dryness in order to avoid rearrangement of the osidic residue caused by the concentrated mineral acid produced.
C.1.7. Gamma irradiation of thymidine bromohydrin in the presence of $^{18}\text{O}_2$.

Trans-(+)-thymidine bromohydrin (400 mg) was dissolved in bidistilled water (8 ml, 0.15 M). The sample was degassed three times under vacuum and saturated with $^{18}\text{O}_2$ (isotopic purity 99.8%) and rapidly frozen in liquid nitrogen before being irradiated for 72 hours (total dose 0.81 MGy). The sample was brought to room temperature and rapidly injected on to a pre-equilibrated reversed phase column using water-methanol (80:20 v/v) eluent. The fraction eluted between the solvent peak and the bromohydrin peak was evaporated to a small volume and the mixture resolved by analytical reversed phase HPLC using water as the eluent (see Section C.1.6).

C.1.8. Gamma radiolysis of thymidine bromohydrin in frozen $D_2O$ solution.

The methodology was identical to that outlined in Section C.1.6. above except that $D_2O$ (99.8% isotopic purity) was used as the solvent.

D. GAMMA IRRADIATION OF 2'-DEOXYCYTIDINE IN FROZEN AQUEOUS SOLUTION.

D.1.1. Gamma irradiation of 2'-deoxycytidine hydrochloride salt.

2'-Deoxycytidine hydrochloride salt (7.92g) was dissolved in bidistilled water (150 ml, 0.2 M) and the resulting solution degassed by passage of nitrogen for 10 minutes. The sample was subsequently frozen rapidly in liquid nitrogen before being irradiated at 196 K for 112 hours (total dose 1.25 MGy). The solution was slowly annealed to room temperature and evaporated to dryness. The residue was taken up in a minimum of hot ethanol and the non-degraded 2'-deoxycytidine hydrochloride removed by successive precipitations and filtrations, down to a volume of 4 ml.

D.1.2. Product isolation.

The final filtrate was evaporated to dryness and taken up in bidistilled water (4 ml). The resulting solution was injected on to a semi-preparative reversed phase HPLC column using water as the eluent (Appendix A.11).

The chromatogram gave seven fractions which were shown to contain the following products.

Fraction A.11.1 ($k'$ 1.14).

Cytosine.
2-deoxy-D-ribono-1,4-lactone.
Fraction A.11.2 (k' 1.43-2.00).
5,6-dihydrouracil.
(5'R6R) 5',6-Cyclo-5,6-dihydro-2'-deoxyuridine (Isomer IV).

Fraction A.11.3 (k' 2.43).
(5'R6S) 5',5-cyclo-5,6-dihydro-2'-deoxyuridine (Isomer III).
1-(2-deoxy-α-D-erythro pentopyranosyl)-5,6-dihydrouracil.

Fraction A.11.4 (k' 2.86).
(5'S6S) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (Isomer I).
(5'S6R) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (Isomer II).
2'-deoxycytidine.

Fraction A.11.5 (k' 3.29-5.29).
1-(2-deoxy-α-D-erythro pentofuranosyl)-5,6-dihydrouracil.
1-(2-deoxy-β-D-erythro pentofuranosyl)-5,6-dihydrouracil.

Fraction A.11.6 (k' 5.71).
1-(2-deoxy-β-D-erythro pentopyranosyl)-5,6-dihydrouracil.

Fraction A.11.7 (k' 8.00).
2'-deoxyuridine.

Cytosine.

Fraction A.11.1 was injected onto a semi-preparative silicagel HPLC column using Solvent II eluent at a flow-rate of 3 ml/min. The peak eluted at k' 1.00 was collected and evaporated to dryness. The white amorphous residue (5 mg) gave an identical 1H NMR spectrum to a commercially obtained sample of cytosine.

2-deoxy-D-ribono-1,4-lactone.

The peak eluted at k' 4.36 from the silicagel chromatography of fraction A.11.1 was collected and evaporated to dryness. The residue (2 mg) was shown to be 2-deoxy-D-ribono-1,4-lactone by comparison of its 1H NMR spectrum with that reported in the literature (159).
5,6-dihydrouracil.

The second fraction from the reversed phase chromatography of the degradation product mixture (A.11.2) was chromatographed on silicagel and the first peak (k' 1.50) was collected and evaporated to dryness. The resulting white residue (4 mg) was shown by 1H NMR to be 5,6-dihydrouracil by comparison with that recorded for a commercially obtained sample.

(5'R6R) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (Isomer IV).

The peak eluted subsequent to that of 5,6-dihydrouracil was collected and upon evaporation to dryness yielded 5 mg of an amorphous white solid (k' 2.64). On the basis of 1H NMR and EI mass spectrometric analyses, the product was assigned the structure (5'R6R) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine.

1H NMR spectrum: see Table II.3

EI MS spectrum:

m/z (rel. abundance) 228 (3.6 M); 169 (3.2); 167 (4.4);
155 (6.7); 114 (30.0); 113 (B);
98 (11.4); 84 (10.5); 73 (35.7); 70 (39).

(5'R6S) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (Isomer III).

Peak A.11.3 from the reversed phase HPLC of the degradation mixture was evaporated to dryness and rechromatographed on a semi-preparative HPLC column using Solvent II. The first peak (k' 1.43) was collected and evaporated to dryness yielding 3 mg of an amorphous white solid. The structure was assigned as (5'S6R) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine on the basis of 400 MHz 1H NMR and EI mass spectrometric data.

1H NMR: see Table II.3

EI mass spectrum:

m/z (rel. abundance) 228 (1.4 M); 169 (0.8); 167 (5.7);
155 (2.0); 114 (14.3); 113 (B);
98 (8.6); 84 (2.8); 73 (20.0); 70 (29.5).

1-(2-deoxy-α-D-erythropentopyranosyl)-5,6-dihydrouracil.

The second peak from the above injection (k' 3.43) was collected and evaporated to dryness. The residue (8 mg) was identified as 1-(2-deoxy-α-D-erythro pentopyranosyl)-5,6-dihydrouracil on the basis of 1H NMR and mass spectrometric analysis.
'H NMR spectrum: see Table II.1

EI mass spectrum:
m/z (rel. abundance) 156 (21.7); 143 (43.4); 141 (23.6);
116 (9.4); 115 (38.2); 114 (16.9);
133 (12.3); 100 (37.7); 99 (35.8):
98 (100); 97 (10.4); 73 (34.9);
70 (11.3); 58 (10.4); 57 (11.3); 56
(20.8); 55 (17.8); 45 (13.2); 44
(19.8); 43 (15.1).

DCI mass spectrum: m/z 231 (MH+); 248 (MNH4+).

(5'S6S) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (Isomer I).

Injection of the fourth reversed phase fraction (A.11.4) on to a silicagel column yielded three peaks, the first of which (k' 1.40) was collected and evaporated to dryness. The white amorphous solid obtained (18 mg) was analysed by one-dimensional 'H and 13C NMR and two-dimensional heteronuclear correlation spectroscopy in D2O and assigned the structure (5'S6S) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine.

'H NMR: see Table II.3

'3C NMR (D2O, TMS) δ ppm: 40.2 (t, C2'), 50.1 (d, C6), 68.1 (d, C3'), 68.5 (d, C5'), 83.4 (d, C1'), 85.6 (d, C4').

(5'S6R) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (Isomer II).

The second peak from the above separation (k' 1.55) was collected and evaporated to dryness to yield 5 mg of an amorphous white solid. The structure was assigned as (5'S6R) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine on the basis of its 'H NMR data.

'H NMR spectrum: see Table II.3

The final peak eluted at k' 11.4 was evaporated to dryness and shown to give a 'H NMR spectrum identical to that of a commercially obtained sample of 2'-deoxycytidine.

1-(2-deoxy-α-D-erythropentofuranosyl)-5,6-dihydrouracil.

The fifth reversed phase fraction was evaporated to dryness and reinjected on to a silicagel column using Solvent II eluent. The first peak (k' 3.70) was evaporated to dryness yielding 12 mg of an amorphous white solid. The residue was analysed by 'H NMR and EI mass spectrometries and assigned the structure 1-(2-deoxy-α-D-erythro pentofuranosyl)-5,6-dihydrouracil.
EI mass spectrum: m/z (rel. abundance) 194 (100); 169 (82.5); 156 (50.0); 144 (29.4); 141 (51.5); 117 (38.2); 115 (73.5); 114 (45.6); 100 (38.2); 99 (35.3); 98 (85.3); 96 (36.8); 84 (29.4); 73 (69.1); 57 (29.4); 56 (80.9); 55 (42.6); 45 (45.6); 43 (44.6); 42 (63.2).

DCI mass spectrum: m/z 231 (MH⁺); 248 (MNH₄⁺).

The second peak (k' 4.05) from the silicagel HPLC of A.11.5 was collected and upon evaporation to dryness yielded 23 mg of a white amorphous solid. From the EI mass spectrum and the ¹H NMR spectrum in D₂O the product was identified as l-(2-deoxy-β-D-erythropentofuranosyl)-5,6-dihydrouracil (5,6-dihydro-2'-deoxyuridine).

²⁷H NMR spectrum: see Table II.1

EI mass spectrum: m/z (rel. abundance) 194 (86.7); 156 (62.6); 144 (33.7); 142 (94.0); 115 (84.3); 114 (56.6); 100 (45.8); 98 (87.9); 96 (41.0); 85 (32.5); 73 (75.9); 70 (35.9); 58 (26.5); 57 (37.3); 56 (100); 55 (54.2); 45 (45.8); 43 (51.8); 42 (85.5); 41 (39.8).

DCI mass spectrum: m/z 231 (MH⁺); 248 (MNH₄⁺).

Peak A.11.6 was chromatographed on a semi-preparative silicagel column using Solvent II. One peak was observed (k' 1.93). Evaporation of this fraction yielded 6 mg of an amorphous white solid. Analysis by ¹H NMR and EI mass spectrometry provided strong evidence for the structure l-(2-deoxy-β-D-erythropentopyranosyl)-5,6-dihydrouracil.

²⁷H NMR spectrum: see Table II.1

EI mass spectrum: m/z (rel. abundance) 156 (20.9); 143 (47.6); 141 (25.7); 116 (12.4); 115 (38.1); 114 (14.3); 100 (38.1); 99 (31.4); 98 (100); 97 (10.5); 73 (38.1); 70 (13.3); 58 (18.1); 57 (15.2); 56 (38.7); 55 (29.5); 45 (22.8); 44 (44.8); 43 (28.3); 42 (19.0).

DCI mass spectrum: m/z 231 (MH⁺); 248 (MNH₄⁺).
2'-deoxyuridine.

The last peak from the reversed phase HPLC fractionation of the degradation mixture was evaporated to dryness and rechromatographed on a silicagel HPLC column. The peak eluting at k' 1.29 was collected and evaporated to dryness. The product (4 mg) was shown to give a 200 MHz 'H NMR spectrum identical to that recorded for a commercially obtained sample of 2'-deoxyuridine.

D.1.2. Gamma irradiation of 2'-deoxycytidine.

A solution of 2'-deoxycytidine (6.84 g, 150 ml, 0.2 M) was degassed by passage of nitrogen gas for 10 minutes and rapidly frozen by immersion in liquid nitrogen. The sample was irradiated at 196 K for 112 hours at a dose rate of 1.12 MRad/hr (total dose 1.25 MGy) and subsequently allowed to anneal to room temperature. The solution was evaporated to dryness and the undegraded 2'-deoxycytidine removed by successive crystallisations from hot ethanol. The final filtrate (4 ml) was fractionated on a semi-preparative reversed phase HPLC column using water eluent at a flow-rate of 3 ml/min. The following products were isolated by subsequent reinjection of reversed phase peaks on to a semi-preparative silicagel HPLC column using Solvent II eluent. The figures in parentheses are the k' values using the silicagel: Solvent II system.

1. Cytosine (k' 11.3).
2. 2'-deoxy-D-ribo-1,4-lactone (k' 4.36).
3. 5,6-dihydrouracil (k' 1.50).
4. (5R6R) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (k' 2.64).
5. (5R6S) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (k' 1.43).
6. (5S6S) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (k' 1.40).
7. (5S6R) 5',6-cyclo-5,6-dihydro-2'-deoxyuridine (k' 1.55).
8. 5,6-dihydro-2'-deoxyuridine (4.05).
9. 2'-deoxyuridine (k'1.29).

E. GAMMA IRRADIATION OF 2'-DEOXYADENOSINE.

E.1.1. Gamma irradiation of 2'-deoxyadenosine in frozen aqueous solution.

2'-Deoxyadenosine (1.9 g) was dissolved with gentle warming in bidistilled water (150 ml, 0.67 M) and the resulting solution degassed by passage of nitrogen for 10 minutes. The sample was then rapidly frozen in liquid nitrogen and irradiated for 150 hours at 196 K (total dose 1.68 MGy). The solution was annealed and the undegraded 2'-deoxyadenosine removed by successive evaporations and precipitations from water down to a final volume of 4 ml. The solution obtained was evaporated to dryness and taken up in 4 ml of water-methanol (90:10 v/v). The sample was fractionated on a semi-preparative reversed phase HPLC column using the same solvent as eluent. Fractions eluted late using this system were rechromatographed directly on a silicagel HPLC column using Solvent II, those fractions eluted early were rechromatographed on the same reversed phase column using water as the eluent and the fractions thus obtained reinjected onto the silicagel system subsequently.
E.1.2. Product isolation.

8-hydroxy-2'-deoxyadenosine.

The fraction eluted from the reversed phase column after the 2'-deoxyadenosine peak using 90:10 v/v water-methanol eluent was concentrated and reinjected using the same eluent. Only one peak was observed (k' 8.44) which was purified on a semi-preparative silicagel column using Solvent II (k' 5.76). Evaporation to dryness yielded 5 mg of a colourless solid. The product was identified as 8-hydroxy-2'-deoxyadenosine by comparison of its $^1$H NMR features with literature data (124).

The eluent from the reversed phase fractionation leaving the column before the 2'-deoxyadenosine peak (k' 6.22) was evaporated to dryness and rechromatographed on a reversed phase column using water-methanol (95:5 v/v) eluent. Six fractions were collected and subsequently reinjected on to a silicagel column using Solvent II eluent.

Peak A.12.1 (k' 3.67)

(5'R) 5',8-cyclo-2'-deoxyadenosine : k' (Si) 5.00, yield 6 mg.

The product was identified by comparison of the $^1$H NMR (68) and EI mass spectra (124) with those found in the literature.

Peak A.12.2 (k' 6.00).

Adenine : k' (Si) 1.86, yield 45 mg.

The product was identified by comparison of the $^1$H NMR spectrum with that of the commercially obtained (Sigma) authentic sample.

Peak A.12.3 (k' 6.72).

9-(2-deoxy-$\alpha$-D-erythropentopyranosyl)adenine : k'(Si) 5.43, yield 8 mg.

Product identification was made by comparison of the $^1$H NMR spectrum with that reported in the literature (118).

Peak A.12.4 (k' 9.55).

9-(2'-deoxy-$\beta$-D-erythropentopyranosyl)adenine : k'(Si) 3.07, yield 3 mg.

The product gave a $^1$H NMR spectrum identical to that published in the literature (118).
Peak A.12.5 (k' 12.11).

(5'S) 5',8-cyclo-2'-deoxyadenosine : k'(Si) 2.29, yield 3 mg.

'H NMR : see Table III.2

EI mass spectrum :
m/z (rel. abundance) 249 (18.1 M); 190 (11.4); 165 (71.4):
164 (19.1); 136 (30.5); 135 (B); 108
(35.2); 81 (15.2); 54 (14.3); 43 (16.2).

9-(2'-deoxy-α-D-erythropentofuranosyl)adenine :
k'(Si) 3.07, yield 4 mg.

E.2. Gamma irradiation of 2'-deoxyadenosine in frozen D$_2$O solution.

2'-Deoxyadenosine was irradiated under identical conditions to those described in Section E.1.1. with the difference that the irradiation solvent was deuterium oxide at 99.8% isotopic purity.

F. IRRADIATION OF THYMIDINE IN FROZEN AQUEOUS SOLUTION WITH 254 NM LIGHT.

F.1.1. Irradiation conditions.

Thymidine (3.63 g) was dissolved in bidistilled water (150 ml, 0.1 M) and the solution obtained poured into a enamel dish (25 x 15 cm) placed in a low density polystyrene open box containing dry ice. Once frozen, the sample presented a uniform layer of approximately 4 mm thickness. The sample was kept at 196 K throughout the 72 hours irradiation period. The sample was then allowed to anneal to room temperature and evaporated to dryness on a rotary evaporator. The bulk of the undegraded thymidine was removed by successive precipitations from hot ethanol and the final filtrate of 10 ml evaporated to dryness. The residue was taken up in 30 ml of water-methanol (90:10 v/v) and injected on to a reversed phase preparative HPLC column. The eluent was collected in three fractions, the first eluting before the thymidine peak, thymidine being collected in the second, and the third consisting of the methanol washings. Each fraction was processed by successive semi-preparative reversed phase and silicagel HPLC injections.

F.1.2. Products eluted before thymidine.

The fraction eluted from the preparative column before thymidine was evaporated to dryness and rechromatographed on a semi-preparative reversed phase HPLC column using water as the eluent. The eluent was divided into two fractions before that corresponding to the residual thymidine (k' 21.5).
First fraction ($k'_{11.35}$).

The first fraction was evaporated to dryness and the residue rechromatographed on a semi-preparative silicagel HPLC column using Solvent II eluent. The following products isolated. The cyclobutidithymidines were identified by comparison of their $^1$H NMR data with those reported in the literature (61). For the spore photoproducts and the dThd(6-4)dPyo adducts, the analysis was made on the grounds of FAB mass spectrometric analysis and $^1$H and $^{13}$C NMR analyses.

Trans/syn(-) cyclobutidithymidine: $k'(Si) 5.00$, yield $3 \text{ mg}$

Cis/syn cyclobutidithymidine: $k'(Si) 7.50$, yield $10 \text{ mg}$.

Cis/anti(-) cyclobutidithymidine: $k'(Si) 8.33$, yield $15 \text{ mg}$.

dThd(6-4)dPyo Isomer I: $k'(Si) 14.68$, $2 \text{ mg}$.

$^1$H NMR: see Table IV.2

FAB-MS: $m/z 485$ (MH$^+$); $507$ (M + Na$^+$).

UV ($\lambda_{max}, \text{H}_2\text{O}$) 320 nm.

Second fraction ($k'_{16.76}$).

The second broad fraction was evaporated to dryness and rechromatographed on silicagel.

Cis/anti(+) cyclobutidithymidine: $k'(Si) 11.65$, yield $20 \text{ mg}$.

F.1.3. Products eluted after thymidine.

The preparative HPLC fraction eluted after the thymidine peak was evaporated to dryness and rechromatographed on a semi-preparative reversed-phase HPLC column using water-methanol (95:5 v/v) eluent. The five peaks were collected and rechromatographed on a semi-preparative silicagel column using Solvent II eluent.

Peak 1 ($k'_{9.60}$).

Trans/anti cyclobutidithymidine: $k'(Si) 6.08$, yield $8 \text{ mg}$.
Peak 2 (k' 11.48).

$dThd(6\rightarrow4)dPyo$ degradation product: $k'(Si)\ 8.57$, yield 2 mg.

$^1H\ NMR:\ see\ Table\ IV.4$

$FAB-MS:\ m/z\ 485\ (MH^+)\ 507\ (M + Na^+)$

$369\ (MH^+ - dR);\ 243\ (dThd + H^+);\ 117\ (dR + H^+).$

$UV\ (\lambda_{max},\ H_2O)\ 272\ nm,\ 319\ nm.$

Peak 3 (k' 15.3).

$dThd(6\rightarrow4)dPyo\ Isomer\ II:\ k'(Si)\ 10.14$, yield 5 mg.

$^1H\ NMR:\ see\ Table\ IV.2$

$FAB-MS:\ m/z\ 485\ (MH^+);\ 507\ (M + Na^+);$

$369\ (MH^+ - dR);\ 243\ (dThd + H^+);\ 117\ (dR + H^+).$

$UV\ (\lambda_{max},\ H_2O)\ 320\ nm.$

Peak 4 (k' 18.4).

$dThd(\alpha-5)dThd\ Isomer\ I:\ k'(Si)\ 11.30$, yield 2 mg.

$^1H\ NMR:\ see\ Table\ I.2$

$^{13}C\ NMR:\ see\ Table\ I.4$

$FAB-MS:\ m/z\ 485\ (MH^+);\ 507\ (M + Na^+);$

$369\ (MH^+ - dR);\ 243\ (dThd + H^+);\ 117\ (dR + H^+).$

Peak 5 (k' 21.89).

$dThd(\alpha-5)dThd\ Isomer\ II:\ k'(Si)\ 11.70$, yield 2 mg.

$^1H\ NMR:\ see\ Table\ I.2$

$^{13}C\ NMR:\ see\ Table\ I.4$

$FAB-MS:\ m/z\ 485\ (MH^+);\ 507\ (M + Na^+);$

$369\ (MH^+ - dR);\ 243\ (dThd + H^+);\ 117\ (dR + H^+).$
F.2.1. Irradiation of thymidine in frozen D₂O solution with 254 nm light.

Thymidine (3.63 g) was dissolved in deuterium oxide (150 ml, 99 % isotopic purity) and the resulting solution poured into an enamel dish (25 x 15 cm). The dish was packed in dry ice producing a frozen layer of approximately 4 mm thickness. The sample was subsequently irradiated at 196 K with 254 nm UV light for 24 hours. The sample was subsequently thawed and the undegraded thymidine removed by successive precipitations from hot ethanol down to a final volume of 10 ml. The final filtrate was evaporated to dryness, the residue taken up in 30 ml of water-methanol (90:10 v/v) and injected on to a preparative reversed phase HPLC column pre-equilibrated with the same solvent. The fraction eluted after the thymidine peak (k' 4.00) was evaporated to dryness and the residue injected on to a semi-preparative HPLC column using water-methanol (95:5 v/v) eluent.

The peak eluted at k' 18.4 was collected and evaporated to dryness yielding 2 mg of an amorphous white solid identified by ¹H NMR in D₂O as Isomer I of the spore photoprocess (see Table 1.2). The last peak eluted at k' 21.7 was collected and likewise evaporated to dryness. Again the residue (2 mg) was identified by ¹H NMR spectroscopy (Table 1.2) as the second isomer of the spore photoprocess.

G. IRRADIATION OF THYMIDINE AS A THIN FILM WITH 254 NM UV LIGHT.

G.1. Irradiation of thymidine.

Thymidine (1 g) was dissolved in methanol (50 ml) and the resulting solution poured into two enamel dishes (40 x 25 cm) and the methanol allowed to evaporate slowly in a fume cupboard. The films produced were irradiated for a total of 72 hours with 254 nm light. Four times during the irradiation period, the films were redissolved in methanol and re-evaporated. After irradiation, the samples were taken up in methanol (100 ml) and combined, filtered, and evaporated to dryness. The residue was taken up in water-methanol (85:15 v/v) and the resulting solution fractionated on a preparative reverse phase column using water-methanol (85:15 v/v) mobile phase. The fractions obtained were evaporated to dryness for subsequent analytical reverse phase and silicagel HPLC purification.

G.1.1. The fraction eluted before thymidine.

The fraction eluted before thymidine from the reversed phase preparative column was evaporated to dryness. The residue was chromatographed on a semi-preparative HPLC column using water eluent at a flow-rate of 3 ml/min. The eluent was separated into two broad fractions centred at k' 11.0 and k' 15.0. Semi-preparative silicagel HPLC chromatography using Solvent II at 3 ml/min of each fraction yielded the following products. The cyclobutane dimers and the 5R and 5S isomers of 5,6-dihydrothymidine were identified by comparison of their ¹H NMR characteristics with literature data (61).
Peak 1 (k'(RP) 11.0).
(5R) and (5S) 5,6-dihydrothymidine: k'(Si) 2.67, yield 3 mg.
Trans/syn(-) cyclobutidithymidine: k'(Si) 5.00, yield 3 mg.
Cis/syn cyclobutidithymidine: k'(Si) 7.50, yield 15 mg.
Cis/anti(-) cyclobutidithymidine: k'(Si) 8.33, yield 25 mg.

Peak 2 (k'(RP) 15.0).
Cis/anti(+) cyclobutidithymidine: k'(Si) 11.5, yield 23 mg.

G.1.2. Products eluted after thymidine.
The fraction eluted after the thymidine peak from the reversed
phase preparative column was evaporated to dryness and taken up in
water-methanol (90/10 v/v) (2 ml). The resulting solution was injected on
to a semi-preparative reversed phase HPLC column using the latter solvent
as eluent (4 x 500 ul). Three peaks were collected and reinjected on to a
silicagel semi-preparative HPLC column.

Peak 1 (k' 4.23).
Trans/syn cyclobutidithymidine: k'(Si) 6.08, yield 4 mg.

Peak 2 (k' 4.89).
dThd(6-4)dPyo Isomer II: k'(Si) 10.14, yield 4 mg.
\^H NMR: see Table IV.1

dThd(α-6)hdThd Isomer I: k'(Si) 10.66, yield 3 mg.
\^H NMR: see Table I.2
\^13C NMR: see Table I.4

Peak 3 (k' 6.11).
dThd(α-5)hdThd: k'(Si) 11.7, yield 40 mg.
\^H NMR: see Table I.2
\^13C NMR: see Table I.4
Appendix A1: Preparative HPLC fractionation of the thymidine degradation mixture.

Apparatus: Prep LC 500  
Column: C18  
Solvent: Water-methanol (85:15 v/v)  
Detector: Refractometer  
Flow-rate: 100 ml/min  
Load: 1.2 g

Appendix A2: Semi-preparative RPHPLC reinjection of preparative HPLC fraction 1.

Column: 3/8" C18  
Solvent: water  
Detector: refractometer  
Flow-rate: 3.0 ml/min
Appendix A3: Semi-preparative RPHPLC reinjection of preparative HPLC fraction 2.

Column: 3/8" C18
Solvent: water
Detector: refractometer
Flow-rate: 3.0 ml/min
Appendix A4 : Semi-preparative silicagel HPLC injection of fraction A.3.1

Column : 3/8" Si
Solvent : Solvent II
Detector : refractometer
Flow-rate : 3.0 ml/min

Appendix A5 : Semi-preparative silicagel HPLC injection of fraction A.3.2.
Appendix A6: Semi-preparative silicagel HPLC injection of fraction A.3.3.

Appendix A7: Semi-preparative silicagel HPLC injection of fraction A.3.4.
Appendix A8 : Semi-preparative silicagel HPLC injection of fraction A.3.5.

Appendix A9 : Semi-preparative RPHPLC reinjection of preparative HPLC fraction 4 and 5.

Column : 3/8" C18
Solvent : water-methanol (95:5 v/v)
Detector : refractometer
Flow rate : 3.0 ml/min
Appendix A10: Semi-preparative RPHPLC reinjection of methanol washings from Appendix A9.

Column: 3/8" C18
Solvent: water-methanol (90:10 v/v)
Detector: refractometer
Flow rate: 3.0 ml/min
Appendix A.11: Semi-preparative RPHPLC injection of 2'-deoxycytidine·HCl degradation products.
Appendix A12: Semi-preparative RP-HPLC injection of fraction eluting before 2'-deoxyadenosine on reversed phase.

Column: 3/8" C18
Solvent: water-methanol (95/5 v/v)
Detector: refractometer
Flow rate: 3.0 ml/min
GENERAL CONCLUSION

We have presented in this thesis the results of a study whose principal aim was the determination of the chemical modifications induced by the direct effects of ionising radiation on DNA model compounds. To this end, nucleosides were irradiated in frozen aqueous solution at 196 K, conditions under which it is widely accepted that the direct effects prevail. The identification of the major lesions and the mechanistic studies of the processes involved in their formation have allowed us to link the primary radicals, which have been extensively studied in the literature by ESR and ENDOR techniques, to their stable progeny, in some cases clarifying intermediate mechanistic steps.

For the pyrimidine nucleosides, we have confirmed the protonation of the radical anion at C6, and the deprotonation of the radical cation at the methyl group for thymidine and at the exocyclic amino group and probably at the C1' position of the furanose residue for 2'-deoxycytidine. The deprotonation of the thymidine radical cation leads to the formation of the relatively stable 5-(2'-deoxyuridilyl)methyl radical which undergoes a variety of combination reactions with other secondary radical species, in particular with the 5,6-dihydrothymid-6-yl radical formed by hydrogen atom addition to C6, and that formed by deprotonation of the thymidine radical cation at N3. Hydrogen loss from the C5' position of both nucleosides has been shown to be a quantitatively important process. For thymidine, it is clear that excitation plays an important role in the degradation of this nucleoside under the direct effects of ionising radiation and the related products have been shown to be formed by exposure to far UV light under similar experimental conditions.

For 2'-deoxyadenosine, the major lesions involve rearrangement of the sugar moiety and this result is likely to reflect indirectly the importance of restitution processes for the primary purine radicals, and secondly the greater lability of the N-glycosidic bond compared to the pyrimidine nucleosides.

We hope that the identification of products apparently unique to the direct effects will eventually incite further research with the aim of detecting these lesions in cellular DNA in order to better estimate the importance of the direct effects of ionising radiation in vivo.
BIBLIOGRAPHY


