

ABSTRACT OF THESIS

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Title of Thesis Stimulated DNA Synthesis in Human Leucocytes following Mutagen Treatment

DNA synthesis ("unscheduled DNA synthesis") that is not associated with the normal process of DNA replication is stimulated in cells exposed to the mutagenic agents UV light, γ -rays, nitrogen mustard (HN2) and methyl methane sulphonate (MMS) and such synthesis is thought to be part of a "repair" process. In this thesis unscheduled DNA synthesis in human lymphocytes exposed to these mutagens has been studied by following the incorporation of tritiated precursors into the DNA. Peripheral blood lymphocytes are almost all in the G₁ stage of the cell cycle so that DNA replication in lymphocyte populations is minimal and mutagen-stimulated DNA synthesis can be readily detected autoradiographically or through assay of the radioactivity (number of disintegrations) in extracted (TCA-insoluble) DNA. These two methods have been used for quantitative work on the kinetics of the process of stimulated DNA synthesis and also as a means of screening for "repair" -deficient human genotypes. A detailed characterisation of the DNA synthesised was undertaken by isolating the DNA on caesium salt gradients.

1. Kinetics and Temporal Studies

For each of the mutagens, the dose response and temporal pattern of synthesis has been determined by scintillation counting of TCA-extracted samples. In each case synthesis was found to initially increase with dose and, following both types of irradiation, this increase was exponential. At very high dose levels of all the mutagens (except γ -rays where the low dose rate restricted the dose range) the amount of synthesis decreased indicating a possible effect of the mutagens on the "repair" system itself.

An attempt to assess the effects of high doses of chemical mutagens on enzyme activity was made in vitro using the enzymes lactic dehydrogenase and fumerase. Although the activity of these two enzymes was inhibited at very high dose levels, the doses necessary to produce inhibitory effects were very much greater than the dose levels at which the "repair" system was affected.

For estimations of the duration of the process of stimulated DNA synthesis, cells were harvested at various times up to 22 hours after exposure to mutagen. A parallel response was found for UV- and HN2-treated cells; for all doses considered, the reaction was largely completed within 6 hours following exposure. This temporal pattern contrasted with the response following γ -irradiation and MMS treatment where synthesis continued for the whole of the incubation time considered. The initial fast reaction following γ -irradiation has been shown to have the same kinetics as the process that results in the rejoining of radiation-induced single-strand breaks and is completed within the first hour post-irradiation. This is followed by a slower linear incorporation of label. A similar biphasic response was found to occur in cells exposed to MMS treatment.

Consideration of the initial rates of the reactions, following treatments with each of the mutagens, indicates that the unscheduled synthesis of the DNA is an enzyme-limited reaction possibly involving a different limiting enzyme in the case of each mutagen.

2. Characterisation of the DNA Synthesised

These experiments were designed in an attempt to explain the different patterns of synthesis that were found for these mutagens. Using caesium salt density gradients to isolate the DNA, the mutagen-induced synthesis following UV irradiation was shown to be "repair replication" (Painter and Cleaver 1968). This was found to be so for cells in all stages of the cell cycle and the temporal pattern of

"repair replication" resembled that of unscheduled DNA synthesis.

Similarly, after HMS treatment "repair replication" showed a similar temporal pattern to unscheduled DNA synthesis. In both PHA-stimulated and unstimulated (G₁) cells, "repair replication" increased with increasing incubation time post-treatment. With increased incubation time there was some evidence for the presence of relatively long regions of substituted DNA, shown by a slight shift in buoyant density of the newly-synthesised DNA relative to the main DNA band.

Following γ -irradiation, "repair replication" was largely completed within 2 hours post-irradiation and it is suggested that this is equivalent to the initial fast reaction evident in TCA-insoluble cell extracts and equated with single-strand rejoining. Synthesis of longer stretches of DNA is evident as ³H-labelled DNA molecules with a greater buoyant density, but even these are not present more than 7 hours after irradiation and it is suggested that some of the newly-synthesised DNA is not being recovered on the gradients.

Exonuclease degradation of DNA from cells labelled with ¹⁴C-thymidine prior to treatment with mutagen and incubation in ³H-thymidine, confirm that all of the newly-synthesised regions resulting from UV-irradiation are distributed evenly along the DNA strand, consistent with a "repair" process. This also holds for the HN2-induced synthesis.

Following exposure to γ -rays or HMS there is a slight tendency for the ³H to be concentrated at the 5' end of the DNA. It is possible that this represents incomplete repair at some of the damaged sites, or alternatively addition of nucleotides onto broken DNA strands.

3. Screening for "Repair" -deficient Human Genotypes

³H-thymidine uptake by UV-irradiated lymphocytes was estimated autoradiographically and comparisons drawn between cells from individuals with certain genetic anomalies and those from controls matched for age and sex. No reduction in DNA synthesis was evident in lymphocytes from patients with Bloom's or Fanconi's syndromes, both of these conditions are inherited as recessives and are associated with spontaneous chromosome damage and a predisposition to leukaemia.

Reduced levels of induced DNA synthesis were evident in cells from a patient with mediastinal and retroperitoneal fibrosis. This patient had a constitutional chromosomal abnormality - a deleted G chromosome (a "Christchurch" chromosome) but cells from a second individual with a similar chromosomes abnormality did not show a reduced level of unscheduled DNA synthesis.

Cells from a patient with Xeroderma pigmentosum also showed reduced levels of unscheduled DNA synthesis confirming previous results, as did those from an individual with a very low chiasma count at meiosis. This latter finding was consistent with other data associating reduced crossing-over with increased radiosensitivity.

STIMULATED DNA SYNTHESIS IN HUMAN LEUCOCYTES
FOLLOWING MUTAGEN TREATMENT

Judith M. Clarkson B.Sc

A thesis presented for the degree of Doctor
of Philosophy in the University of Edinburgh

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SUMMARY

DNA synthesis ("unscheduled DNA synthesis") that is not associated with the normal process of DNA replication is stimulated in cells exposed to the mutagenic agents UV light, γ -rays, nitrogen mustard (HN2) and methyl methane sulphonate (MMS) and such synthesis is thought to be part of a "repair" process. In this thesis unscheduled DNA synthesis in human lymphocytes exposed to these mutagens has been studied by following the incorporation of tritiated precursors into the DNA. Peripheral blood lymphocytes are almost all in the G_1 stage of the cell cycle so that DNA replication in lymphocyte populations is minimal and mutagen-stimulated DNA synthesis can be readily detected autoradiographically or through assay of the radioactivity (number of disintegrations) in extracted (TCA-insoluble) DNA. These two methods have been used for quantitative work on the kinetics of the process of stimulated DNA synthesis and also as a means of screening for "repair" -deficient human genotypes. A detailed characterisation of the DNA synthesised was undertaken by isolating the DNA on caesium salt gradients.

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I. INTRODUCTION

Many of the deleterious effects of exposure of cells to various kinds of irradiation have been known for over half a century and include inhibition of cellular proliferation, the induction of chromosome structural changes, and initiation of malignant transformation. The most important cellular component controlling these events is DNA and evidence that this is one of the principle sites of radiation damage is given below. Subsequent to the early studies with radiations it was realised that certain chemicals, including alkylating agents, produced effects similar to those produced by ionising radiations and these chemical agents are often referred to as radiomimetic substances for this reason.

The radiation survival curves for the proliferative ability of mammalian cells, both in vitro (Puck & Marcus 1956) and in vivo (Till and McCulloch 1961), have an initial shoulder. This indicates that a certain amount of damage accumulates before a lethal effect is observed and this kind of damage has been referred to as "sub-lethal" damage (Elkind & Sutton 1959). Dose fractionation experiments have shown that this sub-lethal damage is repaired rapidly (Elkind and Sutton 1960; Till and McCulloch 1963) and evidence will be presented to show that this is dependent, at least to some extent, on the "repair" of DNA and involves the resynthesis of a small amount of DNA, a process referred to as unscheduled DNA synthesis (Djordjevic and Tolmach 1967). Unscheduled DNA synthesis has been demonstrated following exposure of mammalian cells to various mutagens and the present thesis is concerned with the induction and characterisation of such synthesis in human cells following their exposure to UV- or γ -irradiation and the alkylating agents nitrogen mustard and methyl methanesulphonate.

The significance of this stimulated DNA synthesis in mutation induction and in malignant transformation is also considered with a view to identifying "repair"-deficient human mutants.

1. Evidence that DNA is the Principle Target for Damage

Through shielding either the nucleus or cytoplasm of Habrobracon eggs exposed to UV-irradiation, von Borstel and Wolff (1955) showed that damage to the nucleus resulted in a much greater reduction in survival as measured by hatchability. Subsequently, partial-cell UV-irradiation experiments on human (KB) cells have shown that DNA synthesis can be specifically inhibited in the region of the nucleus exposed to radiation (Moreno 1971).

Further cytological evidence comes from work on chromosome aberration induction. Ionizing radiation was first shown to produce such aberrations by Koernicke working on Vicia faba, Pisium and Lilium in 1905. Sax (1938) later showed that the frequency of exchange aberrations approximately increases in proportion to the square of the X-ray dose. He postulated that the chromosome strands were broken as a result of the irradiation and when the ends rejoined, a proportion did so with those of a different chromosome resulting in an exchange aberration. This interpretation supported by later work (see Lea 1946) and subsequent experiments by Sax (1939 and 1940), Lane (1951) and Evans (1966) using split doses have led to various ideas on the timing of the rejoining process. Lane's results for the frequency of X-ray-induced aberrations in Tradescantia, with increasing delay between fractions, show an initial drop followed by a peak at 8-12 hours. It is interesting that this pattern is similar to the survival data of Elkind and Sutton (1960) using fractionated X-ray doses on Chinese hamster cells.

Chromosome aberrations are also produced in cells after exposure to UV-irradiation (Swanson 1942, Fabergé 1951, Kirby-Smith and Craig 1957, MacKenzie and Muller 1940, Humphrey et al 1963), nitrogen mustard (Auerbach and Robson 1947, Darlington and Koller 1947, Evans and Scott 1969) and a wide variety of chemical agents (Konzak et al 1965, Kihlman 1966, Evans and Scott 1969, Vogel and Röhrborn 1970).

The importance of damage to nucleic acids for the manifestation of these effects is illustrated in the case of UV light by comparing its absorption spectrum with the action spectrum for inactivation of bacteria (Gates 1929, Hollaender and Claus 1936); killing, mutation and thymine dimer induction in Escherichia coli (Setlow and Boling 1965 & 1970); mutation induction in fungi (Hollaender and Emmons 1941); and chromosome aberration formation in Tradescantia (Kirby-Smith and Craig 1957) and Chinese hamster cells (Chu 1962). All these effects are maximal at wavelengths 250-270m μ , as is the absorption of light by DNA.

One convincing type of evidence implicating DNA is the sensitising effect of incorporated halogenated base analogues. Bacillus subtilis and E.coli cells labelled with bromodeoxyuridine (BUdR) show a marked increase in sensitivity to X-irradiation (Greer and Zamenhof 1957). A similar result is obtained by extracting BUdR-labelled DNA, irradiating in vitro and measuring transforming ability in receptor cells (Szybalski and Lorkiewicz 1962). Studies on BUdR-labelled mammalian cells reveal a similar type of sensitisation to UV-irradiation (Djordjevic and Szybalski 1960) and X-rays (Erikson and Szybalski 1961). On the other hand no sensitisation is evident in E.coli grown in 5-fluorouracil which is incorporated into RNA and not into DNA (Kaplan et al 1961, Kaplan et al 1962).

X-ray sensitivity has also been shown to correlate with total DNA content in a wide range of organisms (Kaplan and Moses 1964). In the case of yeasts many examples are known where sensitivity is proportional to ploidy (Mortimer 1958, Haynes and Inch 1963). However that the situation is not always as simple as this is evident from the work of Laskowski (1962). He showed, for Saccharomyces cerevisiae, that X-ray sensitivity was dependent on the genetic background, and that diploids homozygous for mating type alleles were more sensitive than triploids although higher polyploidies showed increasing sensitivity in all cases. Target size has also been shown to be important in plant cells exposed to ionising radiation. In this case sensitivity is proportional to DNA content, and, more specifically, to interphase chromosome volume, and is independent of chromosome number (Sparrow et al 1963).

The importance of DNA base composition in the sensitivity of bacteria to cell killing by UV light, X-rays and nitrogen mustard (HN2) was demonstrated by Kaplan and Zaverine (1962).

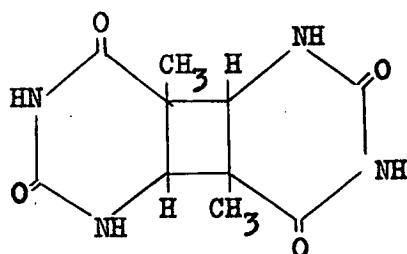
Organisms with DNA rich in GC are more sensitive to killing by X-rays and those whose DNA is rich in AT are more sensitive to UV (Haynes 1963). This difference has also been extended to inactivation of transforming DNA's with different base compositions (Kaplan 1963 quoted by Smith 1964). Doubtless this is a reflection on the type of damage produced by the two agents since there is considerable evidence that a major lesion produced by UV irradiation is the thymine dimer (see below) so that high AT ratios dispose to high UV sensitivity.

2. Damage to DNA by UV-Irradiation and its Repair

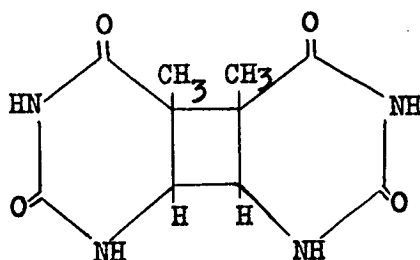
UV light (at 2537⁰Å) is one of the most widely used mutagens

and a great deal is known about the types of damage induced by it in DNA. Much has been learnt about structural changes in DNA by irradiating solutions of bases and nucleotides; such exposures result predominately in hydration and dimerisation of the bases. The quantum yield for purines at $2537\text{\AA}$ is ten times that for pyrimidines (Smith 1966) and therefore at biologically significant doses the latter are probably the more important ones. However the energy absorbed by purines may be transferred to the other bases.

UV-irradiation of thymine in aqueous solution has little effect. However Beukers et al (1958) irradiated a rapidly frozen solution (in which the intermolecular distance is greatly reduced) and found that the characteristic absorption of nucleotides at $260\text{m}\mu$ disappeared. This was restored by reirradiating the thawed solution. In 1960 Beukers and Berend isolated the photoproduct which was shown to be a thymine dimer (TT), and suggested the following structure:



A similar product, reversible by irradiation in aqueous solution, was isolated from DNA of irradiated bacteria by Wacker (1961). A modification of the original structure was suggested by him and agreed upon by Beukers and Berends (1961):



Wavelength studies by Setlow (1961) showed that the action spectrum for the reversal of dimerisation corresponded to the absorption spectrum of the dimer. Subsequently it was established that the photosteady state lies strongly on the side of the dimer at about 275m μ and on the side of the monomer at 235m μ (Setlow and Setlow 1962).

Irradiation of DNA also produces non-photoreversible products probably formed partly by reaction of thymine with other DNA components. These appear to be favoured at higher doses (Wacker 1963).

Irradiation of aqueous solutions of cytidine and deoxycytidine also results in large changes in UV absorption. Cytosine is unchanged but the cytosine residue in cytidine probably adds water across the 5-6 double bond (Shugar 1960). Irradiation of frozen cytosine solutions produces a photoproduct which has the characteristics of UU (Wacker 1963) and is converted to uracil by UV irradiation in aqueous solution. The formation of a uracil dimer is due to simultaneous deamination (Green and Cohen 1957) and has also been demonstrated in irradiated polydeoxyinosinic acid: polydeoxycytidylic acid (Setlow et al 1965) and in bacterial DNA (Dellweg and Wacker 1962). Subsequent DNA replication would therefore result in the replacement of GC base pairs by AU (later AT). Mutations produced in this way have been described for the π II region of T4 phage (Drake 1963).

Uracil was the first of the bases shown to undergo photochemical alteration. In 1949 Sinsheimer and Hastings showed that water could be incorporated across the 5-6 double bond. Later two other photo-products were described (Rörsch et al 1958), including a uracil dimer. The relative amount of each product was found to depend on the dose of UV light. Uracil and thymine in mixed solution form mixed dimers

and this could be important for the cross-linking of DNA and RNA.

More information about the relative biological importance of each of these lesions can be gleaned by irradiating polynucleotide chains. Poly U is randomly coiled at room temperature and the bases are susceptible to both photohydration and dimerisation. Twin-stranded poly (A + U) is helical and the initial reaction is exclusively hydration, dimerisation following partial collapse of the helix by this reaction (Wierzchowski and Shugar 1962). Dimerisation is therefore dependant on prior distortion of the secondary structure of the nucleotide chain. In poly (A + rT) where very few stable hydrates are formed, the helix is more radiation resistant and a pronounced lag precedes dimerisation (Shugar et al 1966).

Cross-linking of the DNA to proteins has been demonstrated for E.coli (Smith 1962); murine lymphoma cells and salmon sperm (Alexander and Moroson 1962); and HeLa cells (Habazin & Han 1970).

Partial denaturation of DNA has been demonstrated by a decrease in the thermal melting point (Marmur and Doty 1959; Marmur et al 1961; Wierzchowski and Shugar 1962). This effect shows much less reversibility by light of short wavelength (Setlow and Carrier 1963).

By measuring sedimentation coefficients, Marmur et al (1961) found little evidence for DNA chain breakage after UV irradiation. Freidfelder and Davison (1963) also consider this to be an infrequent consequence of UV exposure at levels giving a large amount of lethality and found no detectable chain breakage at 1% survival of phage T7. They also found no cross-linking of strands at this level of survival. At very high doses (greater than 10^4 ergs/mm) Baghi (1969) obtained double-strand breaks and cross-linking.

Of the variety of lesions described above, the predominant one resulting from UV irradiation of DNA is the thymine dimer. In stationary bacteria irradiated with increasing doses of UV light, the number of thymine dimers increases. UV sensitivity varies during the cell cycle, increasing during log phase, and using a constant UV dose, again survival can be correlated with dimer formation (Wacker 1963). Setlow et al (1963) showed that UV-induced thymine dimer formation was associated with the inhibition of DNA synthesis in E.coli and in resistant strains synthesis is resumed as the dimers disappear. In Bacillus subtilis the major photo-product of thymine in irradiated vegetative cells is the dimer, but in the spores, which are very resistant, no dimers are produced (Donnellan and Setlow 1965).

Studies on UV-induced damage in bacteria have shown that damage to DNA is subject to repair by the cell. Three types of repair have been characterised: Photorepair (photoreactivation); Excision repair and Recombination repair.

(a) Photoreactivation A post-irradiation treatment found to have a very dramatic effect on survival was described by Kelner (1949) and Novick and Szilard (1949). Incubating irradiated bacteria in the light of a projection lamp caused a constant reduction in the effective dose of approximately 60% compared with cells incubated in the dark. Novick and Szilard found a similar reduction in mutation frequency. This phenomenon is most usually referred to as "photoreactivation" and was defined by Jagger (1958) as "the restoration of UV radiation lesions in a biological system with light of wavelength longer than that of the damaging radiation". The blue-green and near-UV regions of the spectrum are most effective, e.g. maximum

for S. griseus at 450m μ (Kelner 1951) and for E.coli at 350m μ (Jagger and Laterjet 1956), and result in enzymatic splitting of dimers (Wulff and Rupert 1962; Setlow and Setlow 1963). Monomerisation also occurs to some extent at shorter wavelengths, dimers absorbing strongly at 235m μ (Setlow and Setlow 1963).

Although photoreactivation has been found in some eukaryotes including vertebrates (Cook and McGrath 1967; Brunk and Hanawalt 1967; Regan and Cook 1967; Regan et al 1968; Cook and Regan 1969) there has been only one reported case for its presence in human cells (Roth 1968). The studies by Cook and McGrath (1967) show this phenomenon to be widespread among invertebrates, fish and amphibia, but absent in mammals and erratically distributed among the bird tissues tested, brain being the only adult tissue giving positive results. This would seem to indicate that the system is of little biological importance in higher organisms and its presence in human cells (Trosko et al 1965; Trosko and Isoun 1970), including skin (Cleaver 1966) has been unsuccessfully sought several times.

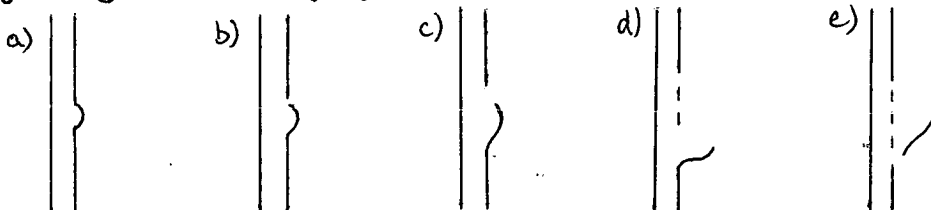
(b) Excision repair Certain strains of E.coli were found to be more resistant than others to UV irradiation following a post-irradiation incubation in the dark (Setlow et al 1963), and this led to the idea of a second repair mechanism (Setlow and Carrier 1964; Boyce and Howard-Flanders 1964). It was found that post-irradiation incubation of the cells in the dark resulted in the disappearance of dimers from the acid-insoluble DNA fraction and their appearance in the acid-soluble fraction in resistant strains. It was postulated that UV-induced lesions were excised and the complementary DNA strand used as a template for resynthesis. Density labelling

experiments showed that DNA replication following UV irradiation of bacteria was non-semiconservative and that the density-label was incorporated into very short segments of DNA (Pettijohn and Hanawalt 1964). This synthesis was found to be independent of protein synthesis, inferring that the necessary enzymes are already present in the cell. It also suggests that this "repair synthesis" is not under the same control as normal DNA synthesis which is limited to one round in the absence of protein synthesis (Maaloe and Hanawalt 1961; Hanawalt et al 1961). This was confirmed by the use of temperature-sensitive DNA synthesis-deficient mutants of E.coli which are found to undergo repair replication at the restrictive temperature (Couch and Hanawalt 1967). Non-semiconservative DNA synthesis also shows greater selectivity for thymine over 5-bromouracil than semi-conservative replication (Kanner and Hanawalt 1968).

The time taken for excision of dimers to occur corresponds to the time required for the resumption of DNA synthesis (Setlow 1964). It requires the presence of an energy source and is inhibited by KCN, caffeine and acridine dyes. Sedimentation of DNA in alkaline sucrose gradients can be used to demonstrate the presence of breaks during the excision process (Setlow 1967a). Extracts from Micrococcus lysodeckticus induce breaks in dimer-containing DNA (Strauss et al 1966) and the DNA retains its sensitivity to the extract until all the dimers are lost. The number of breaks approximates to the number of dimers (Carrier and Setlow 1970) and as judged by the increased rate of digestion by venom phosphodiesterase, these are on the 5' side of the dimer (Setlow 1968). Cruder extracts are also able to specifically excise oligonucleotides containing pyrimidine dimers from irradiated DNA - dimers being removed at twice the rate

of thymine bases (Carrier and Setlow 1966). Takagi et al (1968), Grossman et al (1968) and Kaplan et al (1969) showed that this reaction is dependent on two enzymes, an endonuclease that introduces single-strand breaks in irradiated DNA and a exo-nuclease which acts on double-stranded DNA which has been both irradiated and pretreated with endonuclease. Mutants with reduced endonuclease levels show delayed recovery after UV irradiation (Mahler et al 1971).

It is generally agreed (Setlow 1967) that the "dark repair" of damaged regions of DNA involves three basic steps - excision of the mis-matched region, in some cases including further degradation; resynthesis using the complementary strand as a template; and rejoining of the newly-synthesised region to the DNA backbone:



- a) distortion of DNA due to formation of dimers
- b) endonuclease incision
- c) exonuclease excision
- d) polymerisation using the opposite strand as a template
- e) rejoining of strand by a ligase

An in vitro system with three such stages has recently been described (Heijneker et al 1971). The biological activity of UV irradiated Bacillus subtilus DNA was partly restored after incubation with a UV-specific endonuclease from Micrococcus luteus in conjunction with DNA polymerase and DNA ligase, both from E.coli. Exonuclease III and Kornberg DNA polymerase isolated from E.coli also have the necessary properties for the excision and repolymerisation enzymes (Richardson et al 1964a and 1964b).

It has also been suggested that excision and resynthesis occur simultaneously and a polymerase with both these functions has been described by Kelly et al (1969).

The presence of a "repair" process in mammalian cells, with certain properties similar to the excision repair found in bacteria, was first documented by Rasmussen and Painter (1964). They showed that UV irradiation of HeLa cells resulted in thymidine uptake by all the cells whereas only a percentage of control cells were labelled, corresponding to those in normal DNA synthesis or S phase (in the terminology of Howard and Pelc 1953). This synthesis has since been termed "unscheduled DNA synthesis" (Djordjevic and Tolmach 1967) and it was later shown by Rasmussen and Painter (1964) and Cleaver and Painter (1968) to be a non-semi-conservative synthesis, ³H-thymidine entering both heavy (BUdR-labelled) and light strands of hybrid DNA in patches too small to affect its density. This non-semiconservative synthesis was termed "repair replication" and it could be demonstrated at doses as low as 25ergs/mm². At this dose level 45% of "repaired" DNA can be shown to undergo subsequent semiconservative replication (Rasmussen et al 1970; Painter et al 1970).

UV-induced unscheduled DNA synthesis has also been shown for mouse L-cells (Rauth 1967), human lymphocytes (Evans and Norman 1968a and 1968b), Chinese hamster cells (Cleaver 1969a) and human skin cells in vivo (Epstein et al 1969). The excision of thymine dimers from mammalian cells has also been demonstrated (Klimek 1966; Regan et al 1968; Cleaver and Trosko 1969; Horikawa et al 1968).

(c) Recombination repair In section 4 of this chapter evidence will be presented to show the existence of a second dark-repair process.

This was first described for UV-irradiated E.coli by Howard-Flanders (1969) and is a post-replicative system in which recombination between undamaged regions of daughter and parental strands results in the restoration of an intact DNA template.

Repair replication in mammalian cells is known to be resistant to caffeine (Cleaver 1969b), but the effect of caffeine on mutation frequency and survival in UV-irradiated cells suggests the presence of a second, caffeine-sensitive repair mechanism (Trosko and Chu 1971). Evidence that this is a recombinational type of repair resembling that in bacteria is presented in chapter V, and it is particularly interesting in view of the reduced level of genetic recombination in certain radiosensitive mutants including c3G in Drosophila (Watson 1969) and a desynaptic mutant in barley (Riley and Miller 1966). In this connection it may also be significant that levels of UV-induced DNA synthesis vary in different stages of the mammalian meiotic cycle, being highest during pachytene, the stage at which crossing-over is thought to occur (Kofman-Alfaro and Chandley 1971).

In summary, then, there appears to be at least three types of repair process that enhance survival of UV-irradiated bacteria. Of these, there is little evidence for photoreactivation in mammalian cells, and the evidence for recombination repair is mainly circumstantial. However, UV-induced DNA synthesis does occur, and in view of the fact that this is accompanied by dimer-excision, gives support to the idea that excision repair follows UV irradiation. In the next section DNA degradation and resynthesis following exposure to other mutagenic agents is described and it is suggested that this system of "excision repair" may be a more general error-correcting mechanism.

3. Repair After Other Mutagens

Unscheduled DNA synthesis has also been observed in mammalian cells following exposure to X-rays (Spiegler and Norman 1969; Rasmussen and Painter 1966; Fox et al 1970), and Painter and Cleaver (1967) and Brent and Wheatley (1971) have shown this to be a non-semiconservative type of replication. Studies on post-irradiation degradation of DNA in bacteria (Chapman and Pollard 1969; Shaeffer and McGrath 1965; Emmerson and Howard-Flanders 1965; Pollard and Achey 1964; Miletic et al 1963; McGrath and Williams 1966; Trgovievic and Kucan 1970; Hariharan and Cerutti 1971) adds support to the idea that excision repair follows X-irradiation. Similarly, non-semiconservative DNA synthesis in bacteria (Hanawalt and Hayes 1965) and mammalian cells (Roberts et al 1968; Ayad et al 1969; Roberts et al 1971) follows alkylation. Nitrogen mustard (HN2) treatment of cells results in a period of inhibition of DNA synthesis, the duration of which is proportional to the dose (Harold and Ziporin 1958; Roberts et al 1968). The resumption of DNA synthesis is associated with removal of alkylation products which is more rapid than the rate of overall degradation of DNA (Papirmeister and Davison 1964). This process is lacking in strains of bacteria that are unduly sensitive to alkylating agents (Lawley and Brookes 1965).

Molecular orbital and resonance energy studies show that GC should be a better electron donor and acceptor than AT, and that purine components should be more chemically reactive than pyrimidines (Pullman and Pullman 1959). It is therefore not surprising that guanine is the most reactive site in nucleic acids during alkylation. Brookes and Lawley (1960 and 1961b) have shown that it is the N7

that is preferentially attacked producing 7-alkylguanines. Alkylation also occurs at the N1, N3 and N7 positions of adenine. In native DNA N3 is the principle adenine site with a reactivity one quarter that of guanine (Lawley and Brookes 1963, and 1964). Among the pyrimidines, N1 of cytosine is the sole site and in DNA this is blocked by H-bonding thereby reducing its reactivity (Brookes and Lawley 1962 and 1963).

Bifunctional agents are able to react with two guanine residues producing di(guanine 7-yl) derivatives (Brookes and Lawley 1961a). In this way intra- and interstrand cross-links are formed (Goldacre et al 1949; Loveless and Stock 1959). Consequently considerable distortion of the helix and disruption of DNA replication occurs and this is held to account for the much greater toxicity of bifunctional compared with monofunctional alkylating agents (Brookes and Lawley 1963; Lawley et al 1969).

A slow reduction in molecular weight after alkylation was first shown by Butler et al (1952) and is due to hydrolytic separation of alkylated purines. The rate of chain breakage following alkylation by mustard gas is very slow at neutral pH (Laurence 1963). However after monofunctional agents, such as MMS, depurination is an important part of the reaction resulting in extensive chain breakage (Wahl 1965; Strauss and Wahl 1964; Strauss and Hill 1970).

Strand-breakage is probably the most important lesion resulting from X-irradiation. This can be demonstrated by molecular weight measurements on alkaline sucrose gradients (McGrath and Williams 1966; Veath and Okado 1969). Studies with coliphage show that the number of inviable particles equals the number of double-strand breaks.

As in the case of MMS treatment (Fox and Fox 1969; Prakash and Strauss 1970), molecular weight studies indicate that incubation of the cells following single-strand breakage results in restoration of the DNA molecule (McGrath and Williams 1966; Lett et al 1967; Humphrey et al 1968; Tsuboi and Terasima 1970; Fox and Fox 1970). However it is not easy to say whether this is the final stage in an excision repair process or an entirely different phenomenon (Sawado and Okado 1970). Indeed rejoining enzymes are known to join the 3' hydroxyl termini to 5' phosphoryl termini without the insertion of nucleotides (Zimmerman et al 1967; Lindahl and Edelman 1968).

Strand breakage is probably not the only cause of cell death. Sensitivity of bacteria to X-rays increases with increasing GC content (Kaplan and Zaverine 1962) and is increased by substituted halogenated base analogues. Optical absorption measurements of 5-BUdR solutions after 25-600 Krads showed three times as much damage as in thymine (Lochmann et al 1964). BU sensitisation of T7 is thought to be independent of strand breakage and is eliminated when phage are irradiated in histidine-cysteine in a nitrogen atmosphere where no base damage occurs (Freifelder and Freifelder 1966). However Szybalski (1962) suggests that halogenated bases affect the strength of neighbouring phosphate-ester bonds and that these, together with glucoside linkages are probably the most susceptible to breakage by X-irradiation.

Inter- and intra-strand cross-linking has also been considered as a lesion produced following exposure to ionising radiation. Alexander and Lett (1960) and Alexander and Stacey (1959) found evidence for this in sperm DNA, which is, however, very tightly packed.

There are therefore considerable differences in the types

of damage to DNA following treatment of cells by these mutagens and it seemed probable that this might be reflected in differences in the patterns of unscheduled DNA synthesis induced by them. There is already some evidence for this, and the first two sets of experiments in the present work were aimed at extending these observations by comparing the pattern of thymidine uptake as a result of UV- or γ -irradiation or incubation in HN2 or MMS.

4. Radiosensitive Mutants

A considerable amount of information about any biological phenomenon can be obtained through studies of organisms mutant at one or more loci controlling specific parts of the reactions involved in the phenomenon of interest. One of the first radiation-sensitive mutants was isolated from E.coli strain B (Hill 1958) and is known as B_{S-1}. This was subsequently shown to be unable to excise thymine dimers produced by UV irradiation (Setlow and Carrier 1964) and the number of thymine dimers produced per cell is approximately equal to the number of lethal hits (Setlow et al 1963).

A large number of radiosensitive bacterial mutants have now been described and in a general way most can be placed into one of the following categories (Rörsch et al 1966):

- a) mutants in which the ability to divide after X- or UV-irradiation is reduced (dir⁻ or fil⁻);
- b) UV-sensitive mutants which are also unable to repair UV lesions in bacteriophage (hcr⁻);
- c) UV-sensitive mutants which can however support extracellularly-irradiated phage (uvr⁻);
- d) UV-sensitive mutants that are in addition X-ray sensitive (exr⁻);

- e) UV-sensitive mutants which are X-ray sensitive and also defective in genetic recombination (rec^-).

Two interesting points are immediately obvious from this classification. Firstly, it suggests that cells are either sensitive to UV light and X-rays, or to UV light only. There are, however, exceptions to this generalisation and examples of bacteria which are sensitive to X-rays and not to UV include the temperature-sensitive mutant of E.coli PA22. At the restrictive temperature the UV repair pathway is completely functional, but sub-lethal damage produced by X-irradiation is converted to lethal hits (Fangman and Russel 1971). The second point concerns category (e) (rec^- mutants), in which there is an association between sensitivity to irradiation and a reduction in genetic recombination. The extreme UV- sensitivity of $\text{uvrA}^- \text{recA}^-$ mutants (Howard-Flanders et al 1969) has led to the idea of a second dark repair process not involving the excision of dimers (a process in which the uvr gene is involved (Boyce and Howard-Flanders 1964; Setlow and Carrier 1964). Following UV-irradiation of uvr^- mutants it has been shown that DNA is synthesised in short pieces compared with unirradiated controls or irradiated uvr^+ cells, but that continued incubation results in a resumption to "normal-sized" DNA (Rupp and Howard-Flanders 1968; Smith and Meun 1970). This suggests a post-replicative repair of radiation damage involving recombination between undamaged regions of daughter and parental strands. A similar theory was postulated by Howard-Flanders et al (1968) to explain the increased recombination of episomes transferred from UV-irradiated excision-repair deficient donors. Subsequent experiments involving density label in caesium chloride gradients

have confirmed this and show that the length of the strands involved in the exchange are of the same order as those between mating bacteria (Rupp et al 1971). Radman et al (1970) have shown that this system operates mostly at low doses and that at higher doses the uvr (excision) system is more efficient. However by delaying replication, excision repair is exclusively increased.

Examples of each of the last four types of radiosensitive bacterial mutants have now been isolated in many classes of eukaryotic cells. Mutants sensitive to UV light are known in Saccharomyces (Nakai and Matsumoto 1967; Snow 1967); Schizosaccharomyces (Fabre 1971) and man (Xeroderma pigmentosum- Cleaver 1968), the latter subsequently being shown to be of the hcr⁻ type (Aaronson and Little 1970). Mutants also sensitive to X-rays and therefore comparable with exr⁻ bacteria are known for Neurospora (Schroeder 1970). Radiosensitive and recombination-deficient strains have been described for Saccharomyces (Nakai and Matsumoto 1967; Snow 1968), Neurospora (Schroeder 1970), Ustilago (Holiday 1967), Aspergillus (Fortun 1971), barley (Riley and Miller 1966) and Drosophila c3G (Watson 1969). In view of the wide distribution of such mutations throughout the plant and animal kingdoms, it seems likely that other human genotypes showing abnormal responses to irradiation may exist. An attempt has been made to look for such "mutants" by screening certain types of subjects for a reduction in the capacity of their cells to undergo unscheduled DNA synthesis following UV irradiation.

5. Aims of the Present Work

Most of our knowledge on repair of DNA following mutagenic damage is derived from work on prokaryotes and microbial eukaryotes.

In these organisms there is good evidence for believing that repair of DNA occurs either by photoreactivation, excision repair or recombination repair and that mutations affecting these systems reduce survival. The studies on mammalian cells are largely confined to long-established fibroblast-like cell lines in culture and usually to cells of non-human origin. Mutagenic treatment of these cells induces non-semiconservative DNA synthesis, termed "repair replication" in view of the properties it shares with the DNA synthesis involved in excision repair of bacterial DNA. The demonstration that thymine dimers are excised following UV irradiation adds weight to the argument that this process represents excision repair in mammalian cells. Unlike bacteria, mutagen-induced synthesis can be demonstrated autoradiographically, a process termed "unscheduled DNA synthesis", and this is now equated with "repair" of DNA. In view of the fact that this unscheduled synthesis can be demonstrated in mammalian cells following damage by many agents (in many cases at doses where survival is impossible) and including X-rays where excision of damaged region can not be detected at doses below 10^4 rads (Painter 1968) the term "repair" may be inappropriate in this context. With these factors in mind the present work was initiated with the following aims:

(a) to determine whether unscheduled DNA synthesis and DNA repair could be initiated in "normal" human cells prior to, or in short-term culture, and to determine to what extent these were manifestations of the same phenomenon.

(b) to characterise the DNA synthesis induced by mutagens producing different kinds of lesions in an attempt to obtain a

better understanding of what "DNA repair mechanisms" might exist in human cells.

(c) to look for individuals who are defective in carrying out "DNA repair".

II. MATERIALS AND METHODS

INTRODUCTION

The experiments in this thesis are concerned with estimations of DNA synthesis stimulated by the mutagens UV light, γ -irradiation, MMS and HN2. The cells used throughout, and taken to be representative of those in the body, are freshly-drawn human white blood cells.

Following leucocyte separation and mutagen treatment, the cells were incubated in medium containing radioactively labelled precursors of DNA. In most cases ^3H -thymidine was used and this has been shown to be specifically incorporated into DNA (Reichard and Estborn 1951; Friedkin *et al* 1956). Its uptake is proportional to the rate of DNA synthesis, thymidine triphosphate pools equilibrating with external precursors within about five minutes (Dendy and Smith 1964; Cleaver and Holford 1965). In the present experiments its uptake was estimated in one of several ways. Where synthesis in cells obtained from several people was being compared, autoradiographs were made and silver grain counts over the lymphocytes were taken to be proportional to the amount of DNA synthesised. In the experiments on kinetics of synthesis (Chapter III), all the treatments in any one experiment were carried out on one sample of cells from one individual and this was divided into equal aliquots for the various treatments or incubation times. The amount of DNA synthesis was then estimated as the number of scintillation counts in trichoroacetic acid (TCA)-insoluble material (Schneider 1945).

More detailed characterisation of the synthesis was carried out by isolating the DNA on caesium salt gradients. Using ^{14}C -labelled thymidine or bromodeoxyuridine (BUdR) as a pre-label or marker, the

DNA bands on the gradient could be located. The "repaired" patches were identified with ^3H so that their position on the gradient relative to these could be determined (Rasmussen and Painter 1966). The incorporation of BUdR into DNA to produce daughter strands of increased buoyant density in CsCl gradients was first shown by Simon (1961) and Chun and Littlefield (1961). The subsequent use of fixed-angle rotors has resulted in a high order of resolution of DNA components and enabled many more samples to be spun simultaneously (Fisher et al 1964; Flamm et al 1966). DNA can be separated from very crude preparations by this technique, the protein precipitating at the surface of the gradient and RNA pelleting, in a fixed-angle head, on the wall of the tube, well away from the point of unloading (Flamm et al 1969).

Peripheral blood leucocytes are almost all in G_1 and are therefore ideal for the study of induced DNA synthesis. However, in order to pre-label the DNA it was necessary to stimulate the cells to undergo normal DNA synthesis. Cells were cultured in medium containing phytohaemagglutinin (PHA), a mitotic stimulant (Hungerford et al 1959; Nowell 1960), and 24 hours after its addition to leucocyte or whole blood cultures, DNA synthesis is initiated in the lymphocytes leading to the first wave of mitosis starting at 42 hours (Bender and Prescott 1962; MacKinney et al 1962). By 72 hours a large percentage of the lymphocytes are in their second division having passed through another S phase (Sasaki and Norman 1966; Heddle et al 1967) and this latter period was found to give a larger incorporation of label.

Once the cells had been stimulated with PHA and had incorporated the ^{14}C label, it was necessary to eliminate as much S phase synthesis as possible in order to detect subsequent induced "repair" synthesis

following exposure to mutagens. Hydroxyurea was first shown by Young et al (1964) to inhibit thymidine incorporation into DNA during S phase. In 1969 Cleaver showed that DNA "repair" synthesis was resistant to hydroxyurea and could be used to selectively inhibit S synthesis. Its action is not fully understood, but it is thought to affect the conversion of ribonucleotides to deoxyribonucleotides (Young and Hodas 1964; Young et al 1967) although this is disputed as the primary site of action (Yarbro 1968). Although less than 1 in 1000 lymphocytes in peripheral blood are in S (Bond et al 1958), hydroxyurea was used in many of the experiments where no prelabelling was involved, as it was found to reduce the background level of counts considerably.

TECHNIQUES

1. Blood Supply and White Blood Cell Separation

For the experiments in Chapters III and IV relatively large quantities of blood were necessary and most samples were obtained from polycythaemic and haemochromatotic patients who were routinely bled as part of their therapy. The blood was collected in blood bottles containing acid-citrate-dextrose and used within 24 hours. For the autoradiography experiments 5-10ml samples were collected in 10ml lithium heparin tubes (Stayne Laboratories Ltd). I am grateful to my colleagues for providing many of the samples used as controls in these experiments. It was not always possible to use the blood immediately especially if it had to be sent by post, however it was not usually more than two days old and both control and experimental samples were always taken on the same day.

Leucocytes were separated as buffy coats following two centrifug-

ations at 1000g for 15 minutes. For larger amounts, the blood was distributed into 25ml universal bottles for the first spin and the buffy coat respun in 10ml plastic tubes (Stayne Laboratories Ltd). Small samples were spun in the collecting tubes and respun in Wintrobe tubes. The cells were then suspended in phosphate buffered saline (Dulbecco A, Oxoid Ltd) for mutagen treatment or added to culture medium. The latter consisted of Eagles MEM (Biocult Ltd) containing 100 units /ml Crystapen, 100ug/ml Streptomycin (both from Glaxo), up to 20% foetal calf serum (Biocult Ltd) and PHA (Wellcome). If the blood had been collected in citrate, 35 units/ml of heparin (Evans Medical Ltd) was added in either case.

2. UV Irradiation

This was always carried out in phosphate buffered saline. Where the cells had been precultured, the medium was spun off and the cells washed once in saline. 5ml aliquots of the resuspended leucocytes (approximately 5×10^6 cells/ml) were transferred to 10cm glass petri-dishes. The cell suspension formed a layer 3-4mm deep and in order to obtain a uniform exposure this was continuously stirred with a magnetic stirrer during irradiation. The cells were irradiated at room temperature ($\sim 20^\circ\text{C}$) from above with UV light at $2537\text{\AA}$ (Hanovia low pressure mercury vapour lamp). The incident dose at the surface of the suspension was measured as $23\text{ergs/mm}^2/\text{sec}$ or $1\text{erg/mm}^2/\text{sec}$ (see below) although the dose absorbed by the cells was probably somewhat less than this. After irradiation, cells given the same exposure were pooled and distributed for incubation. Control cells were handled in the same way except that they were not exposed to UV irradiation.

Calibration of the UV lamp The lamp consisted of a flat spiral tube mounted in a cylindrical metal case 12cm in diameter. Its length was such that the lamp was 28cm from the bench top with a shutter just beneath it. The lamp was calibrated in this position and to obtain lower dose rates was supported by longer tubes and the dose calculated by the inverse square law.

- a) Physical method. A Latarjet dosimeter (Latarjet et al 1953) gave a dose rate of 23 ergs/mm²/sec.
- b) Biological method. A survival curve for E.coli B/r was constructed and compared with that of Haynes (1964). A sample of the cell suspension was streaked out onto agar and a colony picked into 20ml of Oxoid nutrient broth number 2. This was incubated overnight at 37°C, shaking continuously. The cells were then suspended in saline at a suitable concentration and the experiment continued in the absence of light at wavelengths conducive to photoreactivation (see Introduction). 3ml samples were irradiated in glass Petri-dishes for 0, 42, 54, 62, and 70 secs. and suitable dilutions made. 0.1ml of three dilutions for each dose were plated onto Oxoid nutrient broth number 2 with 12.5g/l agar. The plates were incubated overnight in the dark, colony counts (100-500 colonies per plate) made and the survival for each dose estimated. This is shown in fig. II 1 together with the curve constructed by Haynes (1964) using known dose levels.

3. γ -irradiation

The leucocytes were suspended in Eagles medium instead of saline at a concentration of $1-2 \times 10^7$ cells/ml. Ten to twenty ml of suspension were introduced into 25ml glass universal bottles, the number depending on the number of doses used. The cells (except

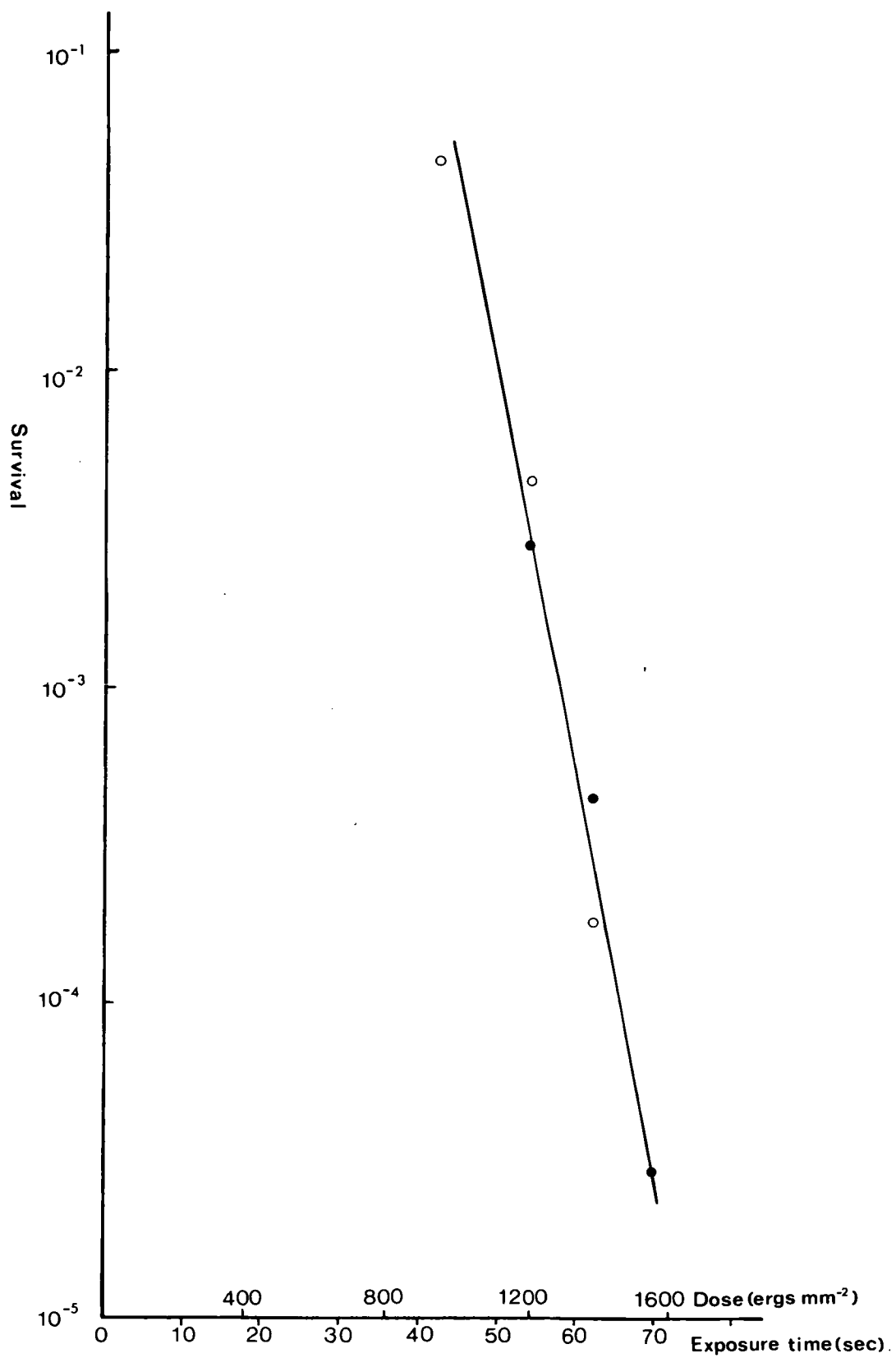


Fig. II 1 The survival of E.coli B/r following UV irradiation 28cm from the source, i.e. as used in many of the experiments. Two estimates were made and are represented by different symbols. The various exposure times used in the calibration are shown together with the equivalent dose level taken from Haynes (1964) for the same survival levels.

for the controls) were irradiated at room temperature (20°C) with ^{60}Co γ -rays at a dose rate of 1000rads/min. They were then distributed for incubation and an equal volume of medium containing twice the concentration of ^3H -thymidine and serum added.

4. Chemical Mutagen Treatments

HN2 was supplied by Boots (Nottingham) and MMS by Kodak Ltd (Kirby, Liverpool).

The cells were usually suspended in saline and distributed into an appropriate number of universal bottles. In a few cases, mostly following preincubation of the cells, they were treated in the same medium. The mutagen, dissolved in saline and diluted within 5 minutes prior to use, was then added at an appropriate concentration to give a total volume of 10ml. After incubation, usually for 1 hour, at 37°C, the cells were removed from the treatment solution by centrifugation and washed twice in saline. They were then distributed and medium added for incubation.

5. Post-mutagen Labelling

Specific details of labelling techniques are described with the appropriate experiments in Chapters III, IV and V. However, basically the technique involves incubation of the cells in Eagles MEM medium containing 100 units/ml Crystapen, 100 μg /ml Streptomycin, 10% foetal calf serum, 10^{-3}M hydroxyurea and a tritiated precursor of DNA.

The experiments in Chapters III and V will be considered first. Following mutagen treatment and subsequent washings, where appropriate, the cells were transferred to 10ml plastic tubes (Stayne Laboratories Ltd) and 3ml aliquots of medium added. For the autoradiography experiments in Chapter V 10 μCi /ml ^3H -thymidine (methyl-T, s.a. 17-25

Ci/mM) was added to the medium and the cells incubated for 2-3 hours at 37°C. Hydroxyurea was omitted as it was possible to distinguish and discount the majority of S phase cells during scoring. A similar medium (but containing hydroxyurea) was used in many of the experiments in Chapter III and the cells incubated for up to 10 hours post-treatment. Since ^3H -thymidine was available throughout the period between mutagen exposure and sampling, experiments of this type are referred to as "continuous labelling" experiments. In most cases comparable experiments were carried out using "pulse labelling". 0.1 $\mu\text{g}/\text{ml}$ of cold thymidine was initially added to the medium and, following a medium change, ^3H -thymidine introduced, usually two hours prior to harvesting the cells.

For the experiments described in Chapter IV larger numbers of cells were necessary and following mutagen treatment various-sized cultures were set up; usually they were 10ml in 25ml universal bottles or for preparative work, 50ml in 100ml medical flats. In many of the experiments a similar medium, containing 10 or 20 $\mu\text{Ci}/\text{ml}$ ^3H -thymidine, was used and the cells "continuously" or "pulse" labelled. Where BUdR uptake during "repair" was being studied, 20 $\mu\text{Ci}/\text{ml}$ deoxycytidine-5- ^3H , 5 $\mu\text{g}/\text{ml}$ BUdR (Koch-Light) and 0.1 $\mu\text{g}/\text{ml}$ fluorodeoxyuridine (FUdR a gift from Dr T. Lennox-Smith, Roche Products Ltd) was added instead of ^3H -thymidine. In "pulse labelling" experiments BUdR and FUdR were added to the "cold" medium prior to the period of ^3H labelling.

All radioactive isotopes except ^{14}C -BUdR (Calbiochem) were obtained from the Radiochemical Centre, Amersham.

6. Autoradiographic technique

Following a 2-3 hour incubation, the medium was spun off using

1000g for 3 minutes and the cells washed twice in saline by adding 4-5ml samples, mixing vigorously and spinning as before. This was followed by two similar washings in 3:1 methanol/acetic acid fixative. The cells were then suspended in a few drops of fixative and dropped onto slides, previously cleaned in alcohol/hydrochloric acid and polished so that the cells flattened onto the slide as the suspension dried out.

Where stated in the text, DNase treatment was applied at this stage. The bottom half of each slide was immersed in 0.02% DNase-1 (Sigma) in 0.002M magnesium sulphate and incubated at 37°C for one hour (Rasmussen and Painter 1964). The slides were then stained overnight in lactopropionic orcein (1g Harleco orcein, 22.5ml propionic acid, 22.5ml lactic acid, 55ml distilled water and refluxed for 2 hours) and washed in 45% acetic acid followed by two rinses in water.

Kodak AR10 stripping film was then applied by floating strips onto water at 24-26°C and lifting the slides up under them. When dry, the slides were stored in a light-tight box in the presence of silica gel at 4°C. After 2-3 weeks, when the mean grain count per cell was about 10-20, the slides were developed in Kodak D19B developer for 10 minutes at 10°C. They were rinsed in water, fixed for 4 minutes in Amfix (Kodak) and washed in water for 10-20 minutes, all solutions being maintained at a temperature below that at which the film was applied. The slides were dried and the number of grains over 100 lymphocytes per slide were counted using a X100 objective and a X10 ocular.

7. TCA Extractions

The radioactive medium was spun off and the cells washed in saline as in the autoradiography experiments, except that three washes were employed, the second containing 1% acetic acid to lyse any remaining red cells. The cells were then transferred to 15ml centrifuge tubes and suspended in ice-cold 5% TCA. They were left at 0°C for, at least 10 minutes (sometimes overnight) and then spun at 10,000g for 10 minutes in a Sorvall RC2-B centrifuge. A second wash in 5% TCA was followed by one in methanol. The precipitate was solubilized in 1ml of "Soluene" (Packard Ltd) and added to vials (Packard) together with 14ml of toluene containing 5g/l PPO (2.5-diphenyloxazole, Koch Light) and 0.3g/l dimethyl POPOP (Thorn Electronics Ltd). They were counted in a Packard Tri-carb scintillation counter using the external standard to estimate the efficiency which was usually 20-30%.

8. Isolation of DNA

Following incubation of the cells the medium was spun off and the cells washed once in saline. They were then suspended in 2-3ml of 10% SSC (Marmur 1961) and one drop of 10% sodium lauryl sulphate added to lyse the cells.

CsCl (BDH ultracentrifuge grade) or Cs_2SO_4 (Merck) and 10% SSC was then added at the required concentration to give 10g gradients. Once the solid had dissolved the mixture was centrifuged at 10,000g for 30 min. in a Sorvall RC2-B centrifuge; in this time a large part of the protein had precipitated at the meniscus (Flamm et al 1969). The solution was then removed with a syringe and 19g needle which was inserted beneath this. 9g was transferred to a 10ml MSE polypropylene tube and overlayed with liquid paraffin. This was spun in a

MSE aluminium fixed-angle rotor at 43,000rpm for 24-40 hours at 20°C. (using an MSE 50 or 65 preparative ultracentrifuge). The gradients were unloaded by hand by piercing the bottom of the tube and pumping the solution out with a peristaltic pump (LKB Instruments Ltd).

This technique is relevant to the experiments in Chapter IV and three types of gradient were employed:

(a) neutral CsCl for preparative work or rebanding experiments. The gradients consisted of 57% CsCl and 20- or 12-drop fractions respectively collected into tubes. The fractions containing the DNA were then identified by pipetting 25 μ l onto Whatmann 3MM filter discs (2.4cm diameter). These were washed in two lots of ice-cold 5% TCA, two changes of 1:1 absolute alcohol/ether and a final rinse in ether (Bollum 1966). When dry, the filters were put into vials containing 2-6ml (Brown and Badman 1961) of scintillation fluid (as before), and counted in a Packard Tri-carb scintillation counter. This was set so that predominately tritium was counted in the red channel (gain 80, discriminator settings 50-420) and ^{14}C only in the green channel (gain 14, discriminators 340-1000). The blue channel was set so that the spillage of ^{14}C into the red channel could be estimated (gain 14, discriminators 340-400).

Appropriate fractions were then pooled and dialysed overnight in $\frac{1}{4}$ " cellulare "Visking" tubing against two changes of 10% SSC (or buffer for subsequent assays).

(b) alkaline CsCl. In the experiments where single-stranded DNA was required, 8 drops of 1M NaOH was added to the gradients to obtain a pH of 12 at which denaturation of DNA occurs (Vinograd *et al* 1963). Due to the increase in buoyant density of single-stranded DNA

(Doty et al 1960) 58.5% CsCl was employed. Following centrifugation 6 drop fractions were collected directly onto Whatmann filters which were washed in TCA as above.

(c) alkaline Cs_2SO_4 . In order to band both normal and BUdR-substituted DNA 37% alkaline Cs_2SO_4 gradients were used as these provide a greater concentration gradient at equilibrium. Fractions were collected as above, either directly onto filters or into tubes for rebanding. For the latter, appropriate fractions were pooled and 37% Cs_2SO_4 (containing a few drops of sodium lauryl sulphate to prevent absorption of DNA onto the walls of the tube. (Flamm et al 1969) added to give a total of 9g. This was centrifuged as before.

9. Prelabelling of DNA

The leucocytes were separated from whole blood as buffy coats (as described previously) and added to the culturing medium. This consisted of Eagles MEM containing 20% foetal calf serum, phosphate buffer pH 7.2 (made up according to Sorenson, see Gomori 1955), 1% PHA and 35 units/ml heparin. Usually 100ml cultures were made up in 200ml medical flats and the cells from 100ml of whole blood added. The cultures were incubated on their side so as to provide the maximum surface area.

For the exonuclease experiments and the preparation of ^{14}C -labelled marker DNA, $0.1\mu\text{Ci/ml}$ thymidine-2- ^{14}C and $0.1\mu\text{g}$ FUdR was added during the first 24 hours. In order to label normal-density DNA in the BUdR experiments ^{14}C -deoxycytosine (uniformly labelled) was used to prevent competition with the BUdR. The latter was added at 48 hours at a concentration of $5\mu\text{g/ml}$ together with $0.1\mu\text{Ci/ml}$ BUdR-2- ^{14}C (Calbiochem) and $0.1\mu\text{g/ml}$ FUdR. At 64 hours this medium

was replaced by that containing 10% foetal calf serum and 2 μ g/ml thymidine and incubation continued for a further 2-3 hours. This was to prevent the formation of molecules labelled with ^{14}C -BUdR plus a small amount of ^3H -thymidine as a consequence of continued S synthesis with the result that tritium counts would appear at a density considerably greater than normal and would be confused with those due to "repair" (Cleaver and Painter 1968).

At 67-68 hours 10^{-3}M hydroxyurea was added to the cultures and left for $\frac{1}{2}$ hour (Fox unpublished). The cells were then prepared for treatment with one of the mutagens.

10. Exonuclease Assays

Following a pre-label in ^{14}C -thymidine and mutagen treatment, the cells were incubated in medium containing 10 μ Ci/ml ^3H -thymidine (as described previously) for 15-16 hours. The DNA was then isolated on CsCl gradients as described in section 8. The fractions containing the DNA were pooled and dialysed in $\frac{1}{4}$ " cellulose "Visking" tubing against two changes of 0.1M Tris-acetate buffer at pH 8.8 (Trisma base-Sigma).

The DNA was usually used the next day but storage at -20°C did not affect the assay. It was denatured by boiling for 8-10 minutes before use. The enzyme (venom phosphodiesterase from Sigma) was dissolved in water. A preliminary experiment with several concentrations showed that its optimum level had an optical density (O.D.) of 0.4 and this was used in all subsequent assays.

Assay mix (Koerner and Sinsheimer 1957):

0.5ml denatured DNA in Tris-acetate buffer

0.15ml 0.3M magnesium acetate

0.5 ml 0.01M ammonium acetate buffer at pH 4.5

0.3ml distilled water

0.1ml enzyme

0.25ml was put into each of six test tubes and incubated for 0, 5, 10, 20, 30 and 40 minutes at 37°C. At these times tubes were removed from the water bath and transferred to ice. Two 0.1ml samples were pipetted onto filters, washed in TCA and counted as before using all three channels.

11. The effect of HN2 and MMS on enzymes in vitro

The fumerase (Sigma) was dissolved in 0.1M phosphate buffer pH 7.4 at a concentration of 4µg/ml. Lactic dehydrogenase (Sigma) was dissolved in 0.03M phosphate buffer at 20µg/ml. 2.7ml aliquots of each were put into 25ml universal bottles. HN2 and MMS were dissolved in the same buffers and one in ten dilutions made. 0.3ml was added to the enzymes to give a range from 10^{-1} to 10^{-5} M MMS and 10^{-2} to 10^{-6} M HN2 and an untreated control for each enzyme. The samples were incubated at 37°C for an hour and then transferred to an ice bath.

Fumerase assay (Racker 1950) 2.8ml aliquots of 0.05M L-malic acid (Sigma) in 0.1M phosphate buffer at pH 7.4 were put into test tubes and 0.2ml of the incubated enzyme added. For each mutagen concentration, samples were incubated for 5, 10, 15 and 20 minutes and the optical density at 240mµ determined using a SP800 spectrometer (Unicam).

Lactic dehydrogenase assay (Worthington Catalogue) The thermostatically-controlled cell holder of the SP800 spectrophotometer was allowed to stabilise at 38°C. 3ml samples of the assay mix was added:

4ml 0.002M β -diphosphopyridine nucleotide, reduced form (Sigma)

4ml 0.01M sodium pyruvate (Sigma) pH 7

108ml phosphate buffer pH 7.4

After a few minutes 50 λ of the incubated enzyme was added and the reaction monitored at 340m μ .

III. THE KINETICS OF UNSCHEDULED DNA SYNTHESIS

INTRODUCTION

Unscheduled DNA synthesis, demonstrated by ^3H -thymidine incorporation, was first described by Rasmussen and Painter (1964) for HeLa and Chinese hamster cells following UV irradiation. They then went on to show that it was a non-semiconservative synthesis induced independent of stage in the cell cycle and roughly proportional to the amount of damage to the DNA. This non-semiconservative synthesis was also demonstrated to occur in cells after their exposure to X-irradiation (Rasmussen and Painter 1966; Painter and Cleaver 1967; Cleaver and Painter 1968). Subsequently it was shown, using several cell types, that the extent of this "repair replication" correlated well with the extent of unscheduled DNA synthesis and it was assumed that both were manifestations of the same process (Painter and Cleaver 1969).

Experiments with inhibitors of DNA synthesis also support the notion that unscheduled DNA synthesis and "repair replication" are manifestations of the same process. Substances such as acriflavine reduce both types of synthesis by a similar amount. Caffeine and hydroxyurea have no effect on either, despite their inhibitory effect on normal semiconservative synthesis, confirming that different enzymes are involved (Cleaver 1969b) as had already been established for bacteria (see Introduction).

Many attempts have been made to relate "repair replication" to increased survival. Cleaver (1969a) demonstrated that a mutant line of Chinese hamster cells having an increased sensitivity to killing by UV light also showed reduced levels of unscheduled DNA synthesis.

He also demonstrated reduced repair replication in UV-sensitive skin fibroblasts obtained from patients with Xeroderma pigmentosum (Cleaver 1968 and see Chapter V). That "repaired" DNA can undergo subsequent replication has been shown following UV- (Evans and Norman 1968a; Rasmussen et al 1970; Painter et al 1970) and X-irradiation (Brent and Wheatley 1971) and it is concluded that this "repair" process has a significant effect on survival at low doses of irradiation. A similar incorporation of label into the DNA of cells not exposed to known mutagens ("undamaged cells") has been demonstrated (Djordjevic et al 1969; Evans et al 1970) and it is postulated that this synthesis of small amounts of DNA represents a general error-correcting mechanism.

"Repair replication" following alkylation of DNA was first demonstrated by Roberts et al (1968) in HeLa cells treated with mustard gas. It has since been shown following exposure of cells to methyl methane sulphonate (MMS) and ethyl methane sulphonate (EMS) (Hahn et al 1968; Ayad et al 1969), N-methyl-(MNNG) and N-ethyl-N'-nitro-N-nitrosoguanidine and N-methyl-N-nitrosourea (MNU) (Roberts et al 1971a). Again attempts have been made to correlate the extent of induced synthesis with increase in cell survival. Using the mutagens mentioned above Roberts et al (1971a) showed that the amount of "repair" synthesis and the loss of alkyl groups was directly proportional to the overall level of DNA alkylation, rather than the extent of the lethal effect. Similar results were obtained by comparing two lines of Yoshida sarcoma cells with very different levels of resistance to cell killing by these mutagens. Increased resistance could not be explained in terms of DNA repair mechanisms (Ball and Roberts 1970).

In this connection it is worth considering the specificity of action of these mutagens compared with radiation. In the Introduction it was established, at least for bacteria, that DNA "repair replication" was independent of protein synthesis and therefore that the necessary enzymes were already present in the cell. UV irradiation at 2570Å would be expected to have relatively little effect on proteins as their absorption spectrum shows that the maximum effect is at 2800Å and even at this wavelength is far less than that of nucleic acids; for serum albumen the absorption at 2600Å is only 1% of that of DNA at the same concentration (Giese 1968). By comparison, it was established some time ago that a significant amount of damage is incurred by proteins following mustard gas treatment although it was appreciated that alkylation of protein was probably not the primary physiological effect of mustards (see review by Wheller 1962). Lawley and Brookes (1968) showed that proteins and DNA isolated from bacterial cells after mustard gas, half sulphur mustard and MMS, were alkylated to a similar extent. Comparable experiments by Roberts et al (1971) with mammalian cells are shown in Table III 1 for doses of mutagen that produces approximately 10% survival in each case. Again, a significant amount of alkylation of protein occurs and in the case of MMS treatment of Chinese hamster cells it exceeds that of DNA.

In considering, then, the effect of chemical mutagens compared with those of irradiation and the ability of the cells to "repair" their DNA, this factor should be taken into consideration. The experiments in this chapter include estimations of the amount of DNA synthesis following different doses of UV light, γ -irradiation, HN2 and MMS. In the case of the two chemical mutagens this has been

Table III 1 The extent of methylation of cellular components following MMS, MNU OR MNNG treatment of Chinese hamster and HeLa cells. Taken from Roberts et al 1971a.

Mutagen	Extent of methylation (μ moles/g cellular component)			
	Chinese hamster cells		HeLa cells	
	DNA	Protein	DNA	Protein
MMS	1.7	3.7	1.0	1.0
MNU	1.2	0.35	0.06	0.025
MNNG	0.8	0.20	0.015	0.007

compared with their inhibitory effect on fumarase and lactic dehydrogenase using an in vitro assay system.

The remaining experiments are concerned with a comparison of the duration and rate of synthesis following these mutagens. A certain amount of data has already been published, mainly for cells irradiated with UV light or X-rays. Evans and Norman (1968a and b) showed that following UV irradiation of lymphocytes, unscheduled DNA synthesis is essentially complete within six hours post-irradiation. However, subsequent studies of Connor and Norman (1971) with polymorphonuclear leucocytes exposed to UV radiation suggested that the synthesis was much more prolonged, a result resembling their earlier data for lymphocytes exposed to ionising radiation (Spiegler and Norman 1969). Results for the duration of synthesis following the latter are also variable depending on the type of cell used and the dose-level studied. Hill (1967) demonstrated unscheduled DNA synthesis for at least five hours, at a continuously declining rate, in mouse L-cells exposed to 8000rads. However following exposure to 4400rads, HeLa cells carried out "repair replication" for only 1-2 hours (Brent and Wheatley 1971), a result similar to that for X-irradiated (150rads) P388F lymphoma cells (Fox et al 1970).

In this study the temporal pattern of synthesis has been investigated in cells considered to be representative of those in the human body i.e. freshly-drawn leucocytes. It was hoped to resolve some of the discrepancies described above, by taking into account different dose-levels, and to include the chemical mutagens MMS and HN2 (mono- and bifunctional alkylating agents respectively) for which there is only one reported time course- P388 cells treated with MMS

(Fox and Ayad 1971). In this chapter unscheduled DNA synthesis has been estimated by ^3H -thymidine incorporation into TCA-insoluble material and in the next chapter these results have been compared with those for "repair replication".

RESULTS

White blood cells were prepared as buffy coats from freshly drawn blood as described in Materials and Methods. They were suspended in saline (plus heparin) for UV-irradiation or chemical treatments. The saline was then spun off and following chemical treatment the cells washed twice in saline. They were then distributed into culture tubes and medium added for incubation. For γ -irradiation the cells were suspended in Eagles medium and then distributed into culture vessels and medium added so that the final concentration of serum, hydroxyurea and thymidine was the same as in the experiments with the other mutagens.

In sections 1 to 4, ^3H -thymidine uptake following exposures to each of the mutagens has been studied over periods of up to 22 hours post-treatment. In each case this has been determined in the continuous presence of ^3H -thymidine and by "pulse" labelling as described previously. At the appropriate harvesting times the samples (usually three per treatment) were washed in saline and extracted with cold TCA as described. In section 5 similar experiments were employed to compare the initial rates (over the first 30 minutes post-treatment) of synthesis in an attempt to identify common rate-limiting enzymatic steps.

Section 6 deals with the effects of these chemical mutagens on

enzyme activities in in vitro assay systems.

1. UV light

³H-thymidine incorporation over periods of up to 20 hours post-irradiation is shown in fig. III 1. A typical result for cells given a 1 minute UV light exposure and incubated in medium containing ³H-thymidine ("continuous labelling") for up to 8 hours is shown. Synthesis is seen to be completed within the first 4 hours following exposure and the amount of label incorporated is some 3 to 4 times that in the unirradiated controls. After 15 seconds exposure and 2 hours "pulse labelling" at different times following irradiation, a similar result is obtained, the rate of incorporation declining over the first six hours, at which time synthesis is virtually complete.

These experiments were carried out with the cells 28cm from the UV lamp. A calibration survival curve covering 4 log decades, where survival is exponential, is shown in fig. II 1 for E.coli B/r irradiated in the same position. The experimental details have been described in chapter II and are comparable to those of Haynes (1964). It may be seen from the graph that 10^{-4} and 10^{-3} survival levels are obtained respectively from exposures of 65.5 and 58 seconds. From Haynes' data, these survival levels are obtained at dose levels of 1450 and 1300 ergs/mm² respectively. Extrapolating these, the dose rate from the lamp is 23 ergs/mm² and is in agreement with that obtained using a Latarjet dosimeter.

To obtain lower dose rates the lamp was supported on longer tubes (65 and 134 cm respectively) and the dose rates calculated using the inverse square law. At these distances, incident doses of 4.2 and 1 erg/mm²/sec were obtained. Using these three positions, a range of

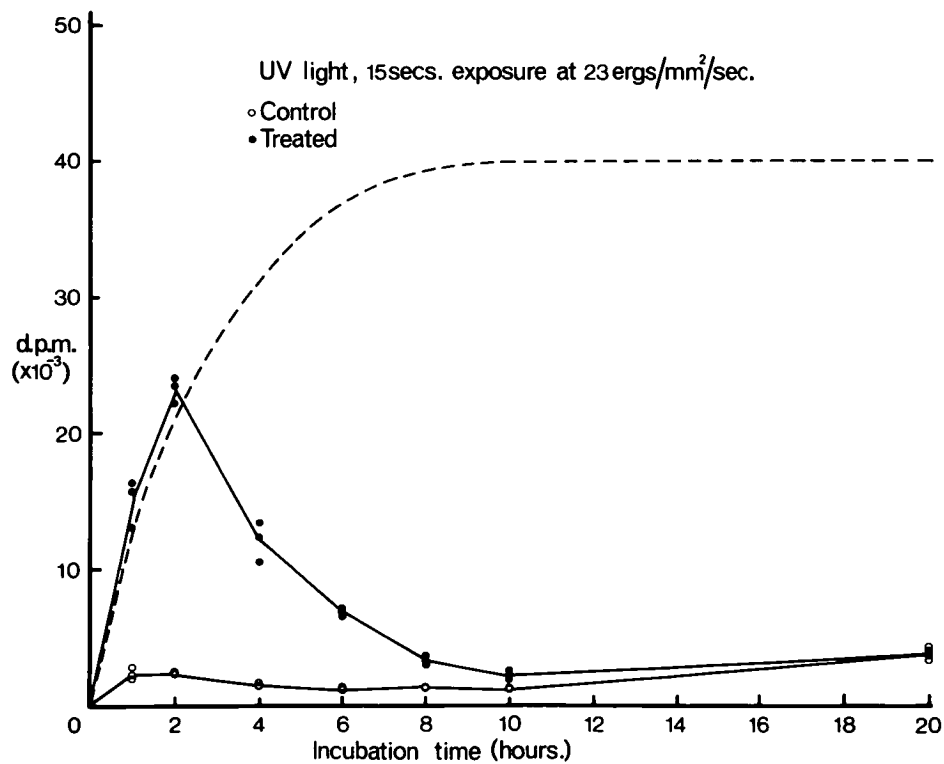
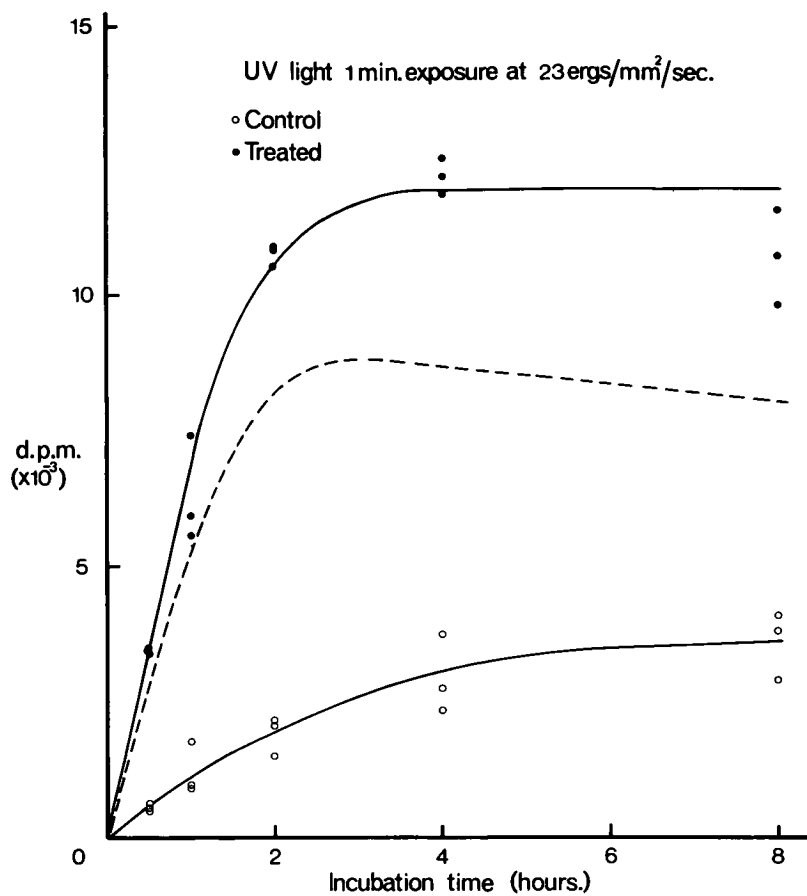


Fig. III 1 Uptake of ^3H -thymidine after exposure to UV light.

Three samples (cultures) were analysed at each time and the dotted line shows net uptake. In the upper fig. a one minute exposure to an incident dose of $23 \text{ ergs/mm}^2/\text{sec}$ (1400 ergs/mm^2) was followed by continuous incubation in "hot" medium. Shown in the lower fig. is the uptake after a similar 15 sec. exposure (350 ergs/mm^2) followed by "pulse"labelling. Dpm values represent uptake of ^3H -thymidine over the two hours prior to sampling, the cells first being incubated in "cold" medium. The incorporation at 20 hours represents uptake from 10 to 20 hours.

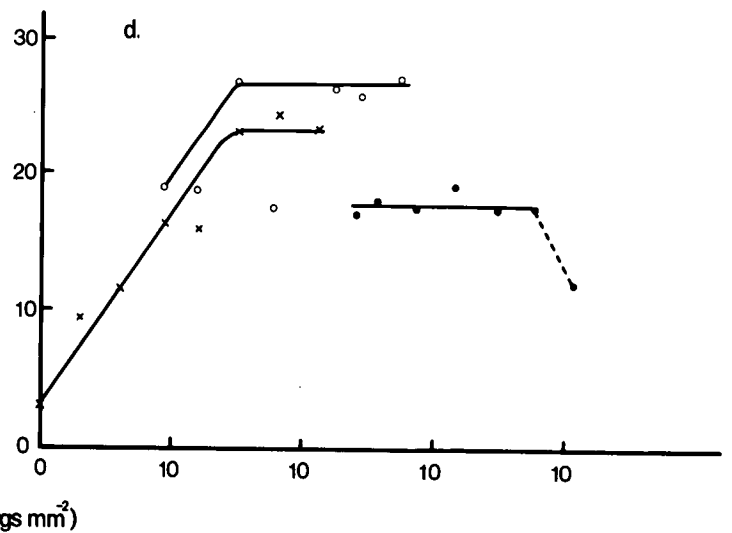
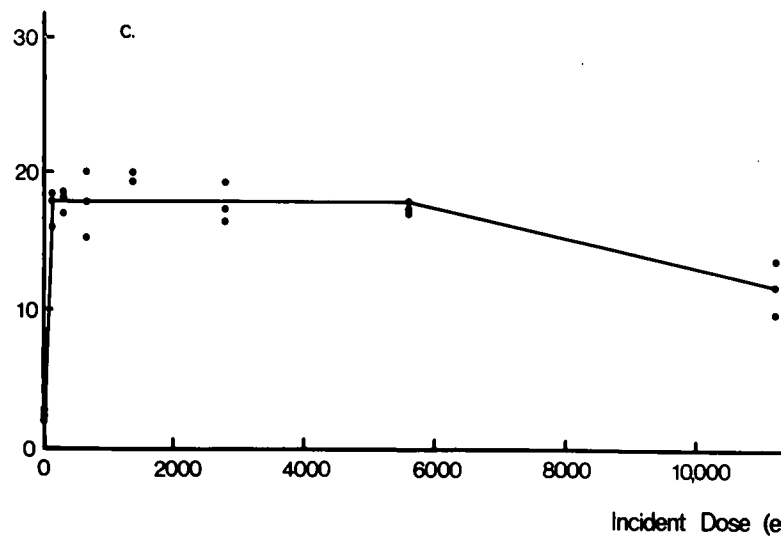
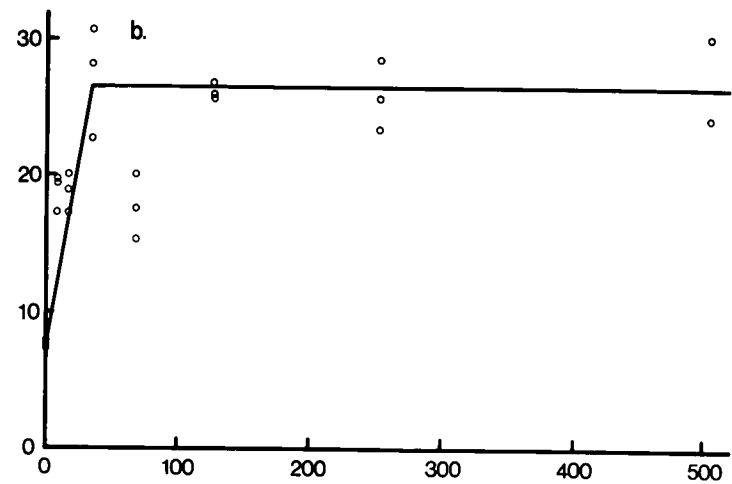
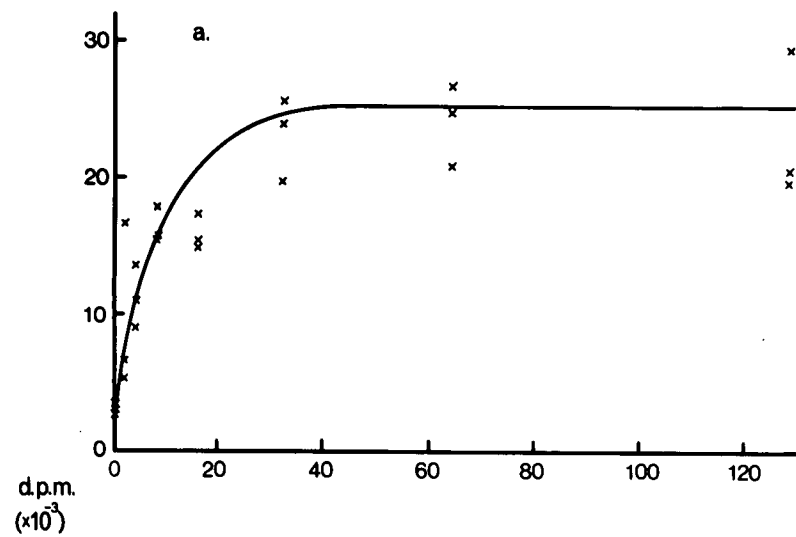


Fig. III 2 ^3H -thymidine uptake during a three-hour incubation following various doses of UV light

- (a) Doses up to 128 ergs/mm^2 given at an incident dose of $1 \text{ erg/mm}^2/\text{sec}$
- (b) Doses from $8\text{--}500 \text{ ergs/mm}^2$ given at an incident dose of
 $4.2 \text{ ergs/mm}^2/\text{sec}$
- (c) Doses from $175\text{--}11200 \text{ ergs/mm}^2/\text{sec}$ given at an incident dose of
 $23 \text{ ergs/mm}^2/\text{sec}$
- (d) The data from each of these experiments plotted on a log. scale

doses from 2 to 10^4 ergs/mm²/sec could be studied. A series of experiments covering this dose range were carried out and in fig. III 2 ³H-thymidine uptake in the first three hours following exposure is shown. Incorporation of label is seen to increase with increase in dose up to exposures of about 40 ergs/mm² and then become dose independent up to 5600 ergs/mm². After 8 minutes at 23 ergs/mm²/sec (incident dose of 11,000 ergs/mm²) the amount of ³H-thymidine incorporated is reduced to about one half of the maximum value. A striking feature of all the dose response experiments with UV light is the very high sensitivity of the system. Considerable incorporation is observed at very low exposures, with 50% of the maximum possible incorporation being obtained at a dose of 4 ergs/mm² and saturation occurring at around 30 to 40 ergs/mm².

It is evident from these data that the time curves of incorporation shown in fig. III 1 were obtained at doses at which synthesis is maximal. In fig. III 3 uptake (determined by 2 hour "pulse labelling") for the first 10 hours post-irradiation is shown for 10, 20, 50 and 100 seconds exposure at an incident dose rate of 1 erg/mm²/sec. The same temporal pattern is obtained at each dose level, the rate being proportional to the amount of damage up to 50 seconds exposure. In each case some 75% of the synthesis is complete in 4 hours and the rate of incorporation falls off very markedly at this time.

2. γ - rays

The results for UV light relating the level of incorporation to time after exposure and shown in fig. III 1 should be contrasted with those for comparable experiments with γ -rays (fig. III 4).

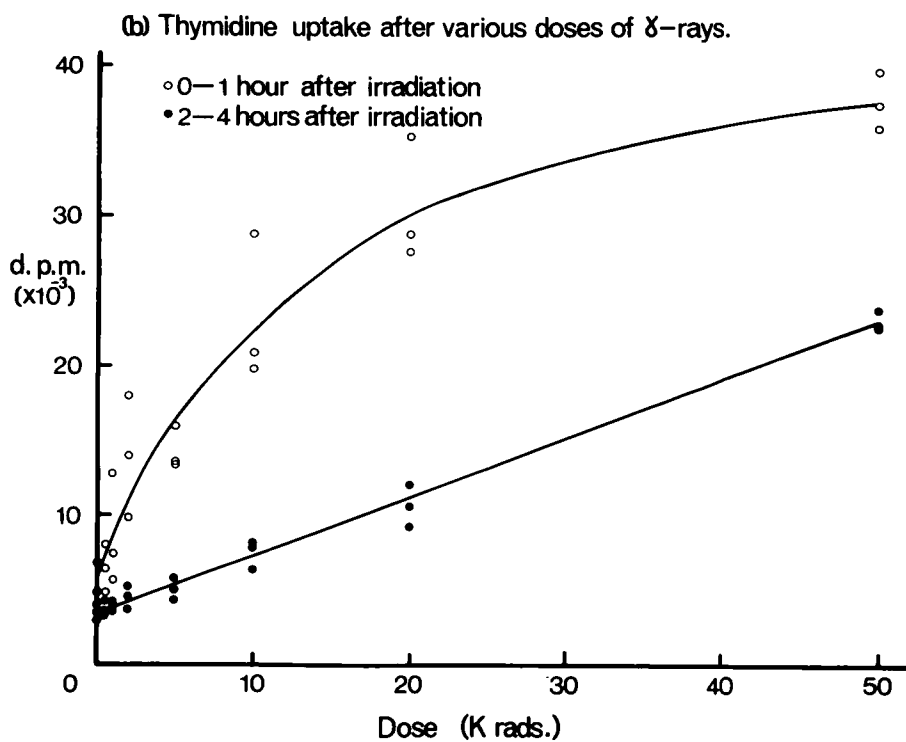
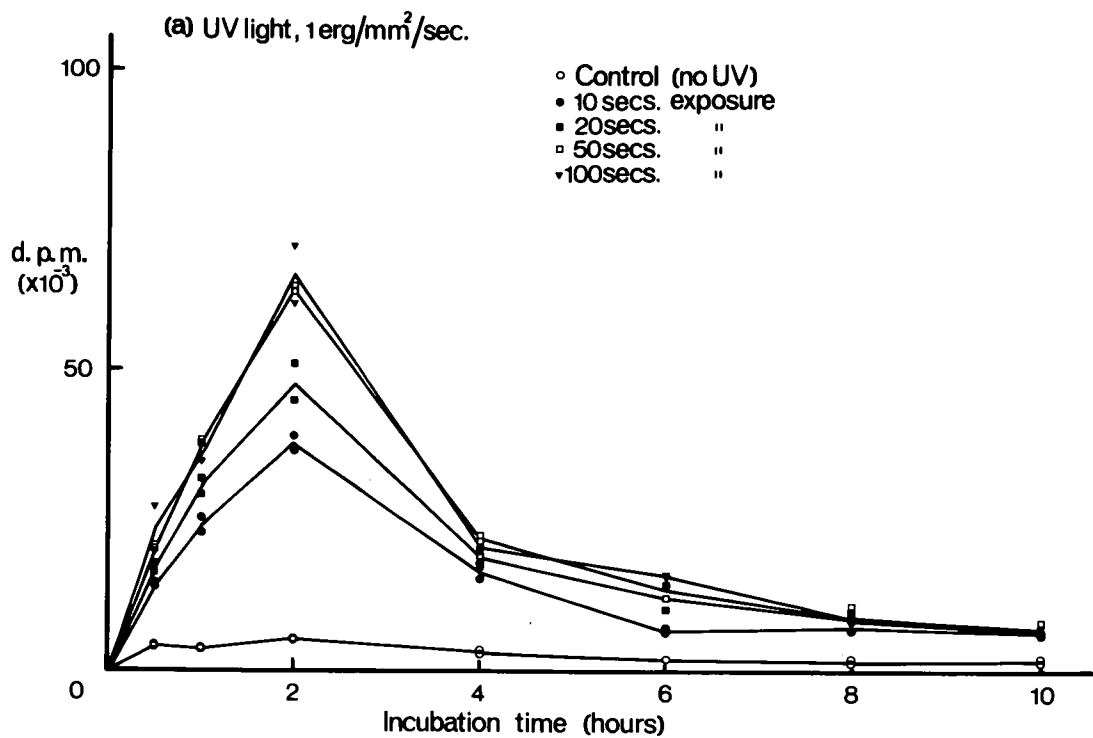


Fig. III 3 (a) ^3H -thymidine uptake after exposure to various doses of UV light up to 100 ergs/mm^2 given at a dose rate of $1 \text{ erg/mm}^2/\text{sec}$.

Two samples were analysed at each time and the dpm values represent uptake of ^3H -thymidine over the two hours prior to sampling, the cells first being incubated in "cold" medium.

(b) ^3H -thymidine uptake after exposure to various levels of ^{60}Co γ -rays at 1 k rad/min .

-o- shows uptake by cells incubated at 37°C during the first hour post-irradiation. -●- cells incubated in "cold" thymidine 0-2 hours and "hot" thymidine 2-4 hours post-irradiation.

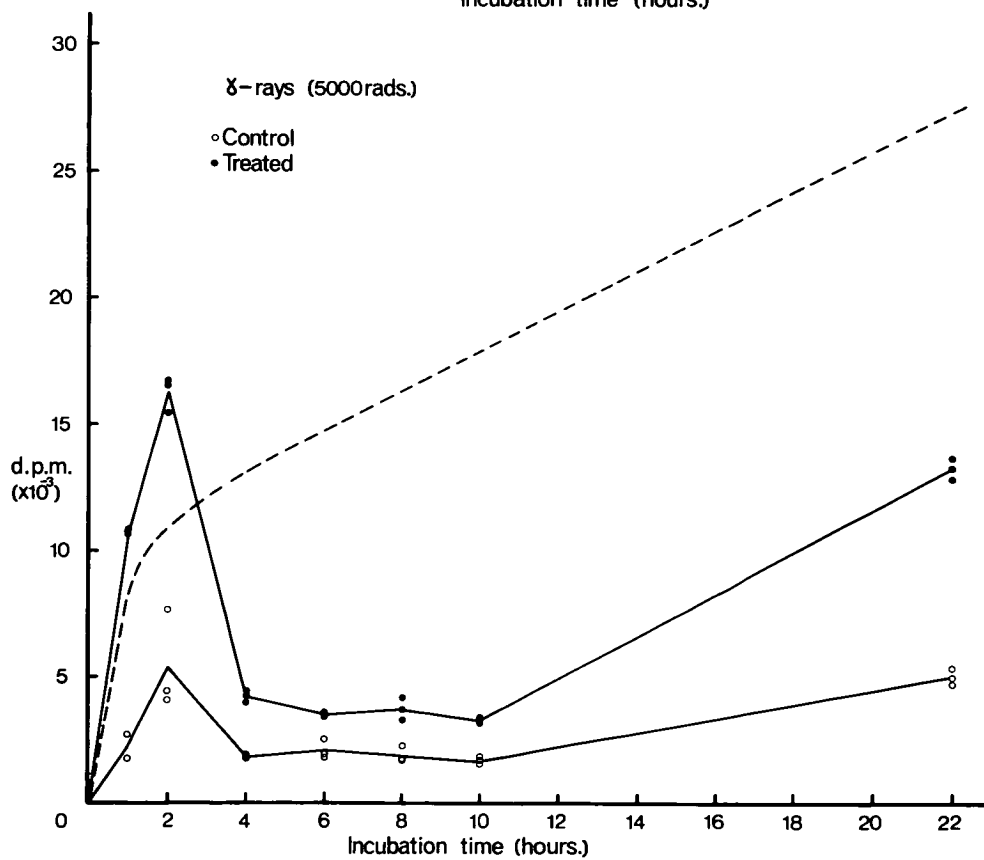
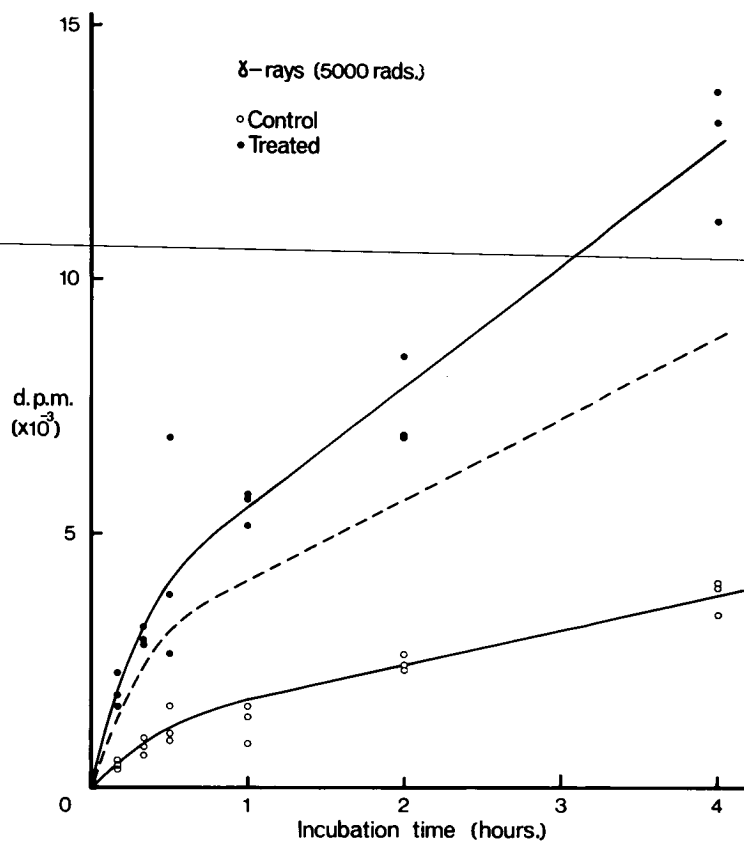


Fig. III 4 ^3H -thymidine uptake after 5000rads of γ -rays

Three samples were analysed at each time, and the net incorporation shown as a dotted line. In the upper fig. the cells were continuously incubated in "hot" medium and in the lower fig. the results of pulse labelling are shown. For the latter the dpm values represent ^3H -thymidine uptake for the two hours prior to sampling (at 22 hours, from 10 to 22 hours), the cells first being incubated in "cold" medium.

In the ^{60}Co γ -ray experiments in each case cells were exposed to 5 k rads and the pattern of incorporation over 20 hours after pulse labelling determined. Unlike the results obtained with UV light, the incorporation continues up to at least 22 hours after exposure, with a constant rate from 2 hours onwards. The initial reaction is more rapid and by fitting a segmented regression to the first set of data, the two components are each shown to be linear with a sharp transition at 36 minutes. The reaction rates are 7732 ± 2977 dpm/hour and 2425 ± 250 dpm/hour respectively; i.e. there is a sudden reduction in the rate of synthesis at 36 minutes to one third of that immediately post-irradiation.

Using these same data an approximate estimate of the number of nucleotides per lesion during this initial fast reaction can be made. The net incorporation during the first 30 minutes post-irradiation

$$= 3000 \text{ dpm} = \frac{3000}{60} \text{ disintegrations/sec.}$$

$$1 \text{ Ci} = 3.7 \times 10^{10} \text{ dis/sec}$$

$$= \frac{3000}{60 \times 3.7 \times 10^{10}} \text{ Ci}$$

$$\text{Specific activity of thymidine used} = 20 \times 10^3 \text{ Ci/mole}$$

$$\text{Amount of thymidine incorporated} = \frac{3000 \times 10^{-3}}{60 \times 3.7 \times 10^{10} \times 20} \text{ moles}$$

$$= \frac{3000 \times 10^{-3} \times 6 \times 10^{23}}{60 \times 3.7 \times 10^{10} \times 20} \text{ molecules of thymidine}$$

$$= 4 \times 10^{10} \text{ molecules of thymidine incorporated}$$

$$\text{The number of cells per sample} = 3-5 \times 10^6$$

$$\therefore \text{number of molecules of thymidine/cell} = 10^4$$

$$\text{GC content of mammalian cells} = 40\%$$

i.e. 30% of the bases in the DNA are thymine and the total number of nucleotides incorporated is therefore 3 times this

i.e. 3×10^4 nucleotides/cell are incorporated into the DNA
 Total nucleotides/cell = $X \times 10^{10}$ (Herskowitz 1967).

$$\therefore \frac{X \times 10^4}{3 \times 10^{10}} \times 100\% \text{ of the genome is replaced} = 10^{-4}\%$$

By measuring ^3H -thymidine uptake by human lymphocytes irradiated with X-rays over the dose range 600 to 2400 rads and assuming a single enzyme system, Spiegler and Norman (1970) estimated that approximately 10 lesions were produced per rad.

Therefore at 5000 rads 3×10^4 nucleotides/cell is equivalent to

$$\frac{3 \times 10^4}{5000 \times 10} \text{ nucleotides/lesion} = 0.6 \text{ per lesion}$$

The uptake of label after a wide range of γ -ray doses at room temperature followed by a constant incubation time at 37°C , was also studied (fig. III 3). A dose rate of 1 k rad/minute and dose levels of 0, 1, 2, 5, 10, 20 and 50 k rads were used. Following exposure half of the cells were incubated in ^3H -thymidine for one hour in an attempt to estimate the dose response for the initial fast reaction. However, at the low dose rates (long radiation exposure times) available the data obtained from high levels of exposure are not particularly informative. For the doses considered, increasing exposure increases the incorporation. This effect is most marked at low doses and the pattern is similar to the exponential increase observed following UV doses. It should be noted however, that a significant amount of synthesis is not evident until relatively large doses (1000 rads) of irradiation are used. The apparent plateauing effect at 50,000 rads may or may not be significant at this

dose level, but if maximum incorporation occurs at this dose level half of this value is not obtained unless 10,000 rads are used. This is in marked contrast to the UV data where one half of the maximum incorporation occurs at 4 ergs/mm². A rough, order of magnitude calculation, excluding factors such as energy transfer between protein and DNA and ignoring the different kinds of damage produced by these radiations, indicates that this discrepancy can be accounted for, to a large extent, by differences in the energy absorbed by the DNA, viz:

Diameter of lymphocyte nucleus = 6-8 microns (Trowell 1958)

$$\text{From this, area of nucleus} = (4 \times 10^{-3})^2 \times \frac{22}{7} \text{ mm}^2$$

$$= 4 \times 10^{-5} \text{ mm}^2$$

$$1 \text{ erg/mm}^2 \text{ incident dose} = 4 \times 10^{-5} \text{ erg/nucleus, most of which is absorbed by the DNA}$$

By definition 1 rad = 100 erg/g

Amount of DNA per cell = 6×10^{-12} g

1 rad is equivalent to $100 \times 6 \times 10^{-12}$ ergs/nucleus

$$\text{i.e. } 1 \text{ erg/mm}^2 \text{ incident dose of UV light} = \frac{4 \times 10^{-5}}{6 \times 10^{-10}} \text{ rads of}$$

$$\text{ionising radiation} = 10^5 \text{ rads}$$

When ³H-thymidine is present 2-4 hours following irradiation a linear increase in incorporation with dose is observed (fig. III 3b). The reaction is therefore directly dependent on the amount of damage induced and may well therefore be controlled by a different enzyme system from that involved in the synthesis during the first hour following exposure.

3. HN2

³H-thymidine incorporation following various incubation periods after a one hour treatment at 37°C with 10⁻⁴M and 10⁻⁵M HN2 are shown

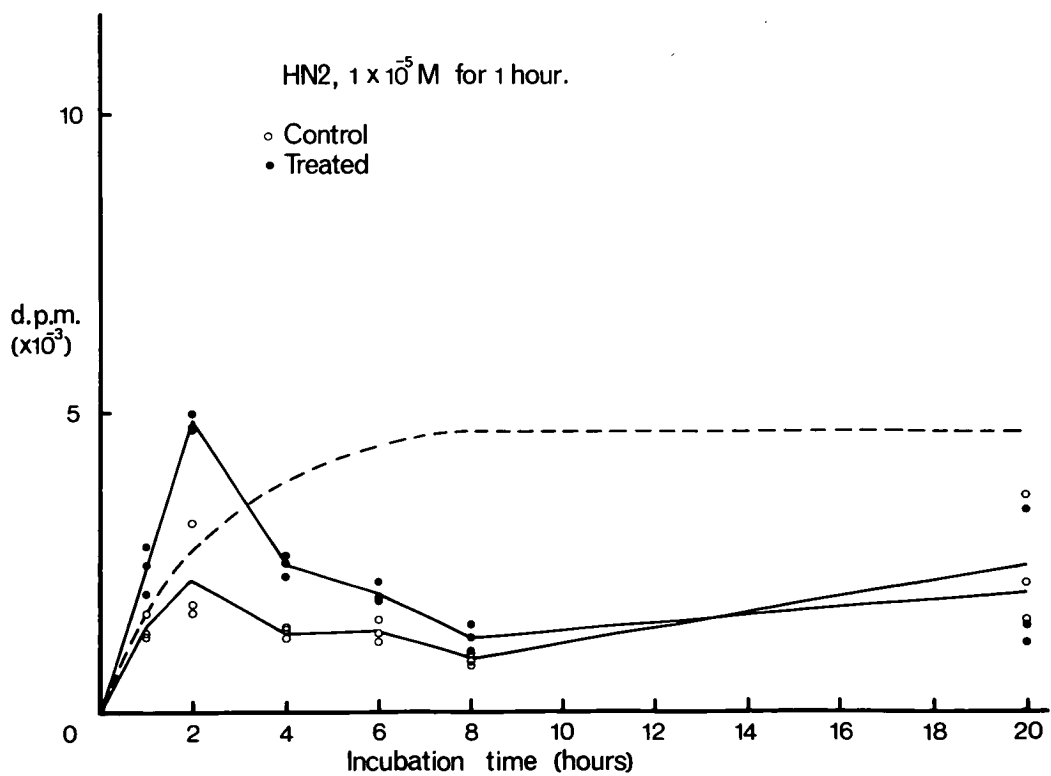
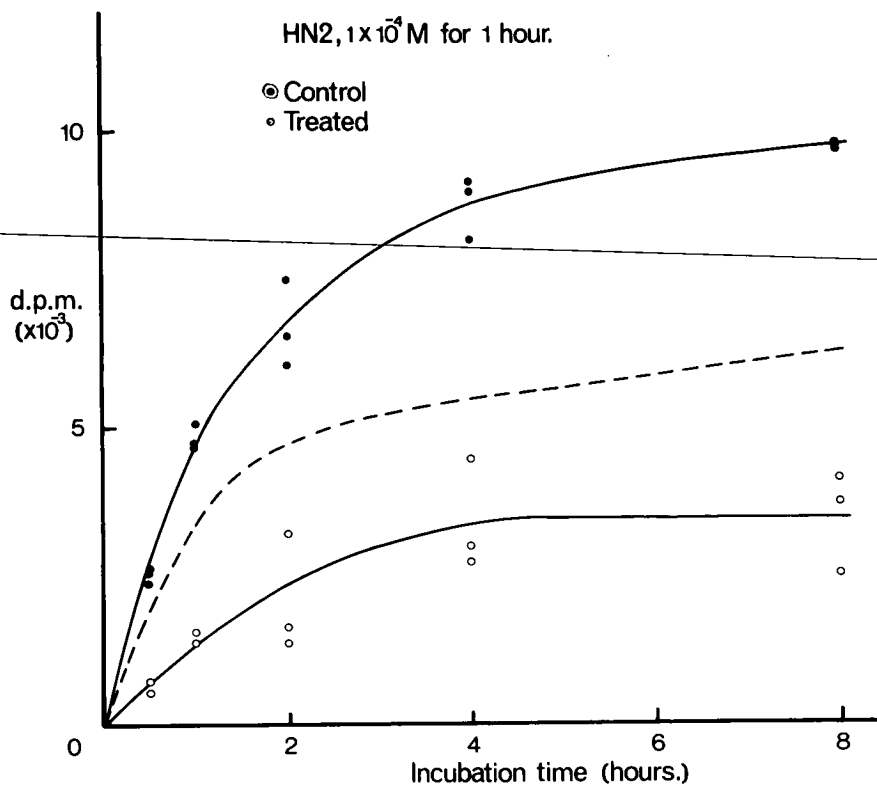


Fig. III 5 ^3H -thymidine uptake following a one hour incubation at 37°C in HN2

Again three cultures were analysed at each time. The upper fig. show the result of continuous incubation in "hot" medium following 10^{-4}M HN2 and in the lower fig. 10^{-5}M HN2 was followed by pulse labelling. Each dpm value represents incorporation over the two hours prior to sampling, the cells first being incubated in "cold" medium. The incorporation at 20 hours represents uptake from 8 to 20 hours. Net counts are shown as before and should be compared with fig. III 1.

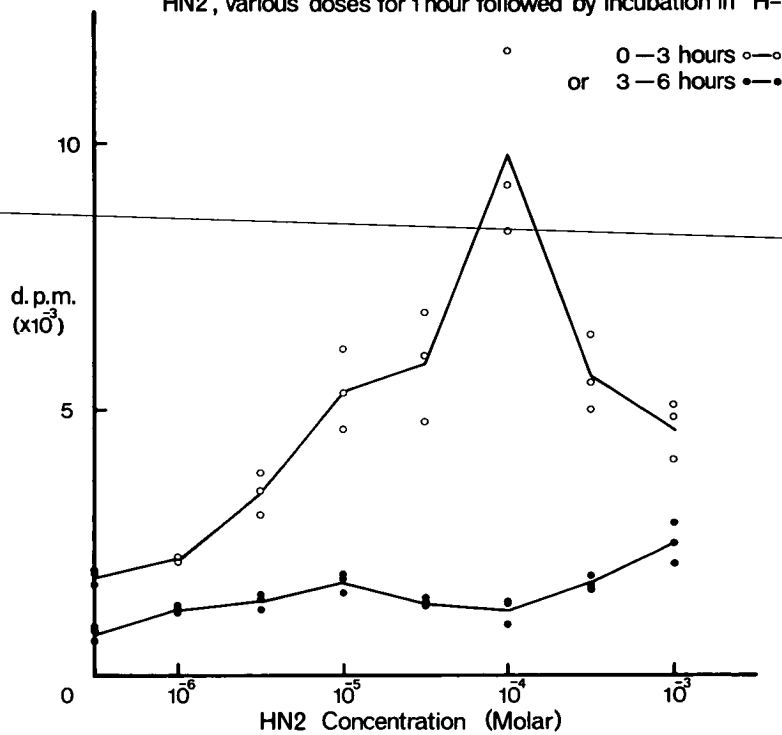
in fig. III 5. The pattern of incorporation very closely resembles that following UV irradiation in that synthesis had been nearly completed by 4 hours. In the first experiment 10^{-4} M HN2 was followed by continuous ^3H -thymidine for up to 8 hours resulting in 3 times the incorporation found in the controls. After 10^{-5} M HN2 and pulse labelling, a similar pattern of uptake occurs, although somewhat reduced in total amount. As with the UV light experiments, there is no further incorporation of label between 6 and 20 hours following exposure (c.f. figs. III 5 and III 2).

The incorporation of label into the DNA of cells following exposure to different concentrations of HN2, and post-incubation for 3 or 6 hours in ^3H -thymidine, is shown in fig. III 6. Uptake increases with increasing dose between 10^{-6} M and 10^{-4} M and then falls at higher concentrations. Similar falls at high dose levels are observed with UV and MMS (c.f. figs. III 2 and III 6). It is evident that the incorporation at all concentrations between the third and sixth hour of incubation following HN2 treatment is small compared with that over the first 3 hours, confirming that the rate of synthesis is independent of dose over the range studied, an independence similar to that found after UV.

4. MMS

Experiments similar to those with HN2 were carried out. In fig. III 7, the uptake of ^3H -thymidine into the DNA of cells treated with 10^{-3} M MMS for 10 minutes and pulse labelled is shown. Uptake continues for at least 21 hours after exposure in a manner similar to that observed after γ -ray irradiation. However, the initial rapid rate characteristic of the γ -ray data is not as marked, although

HN2, various doses for 1 hour followed by incubation in ^3H -thymidine.



MMS, various doses for 1 hour followed by incubation in ^3H -thymidine.

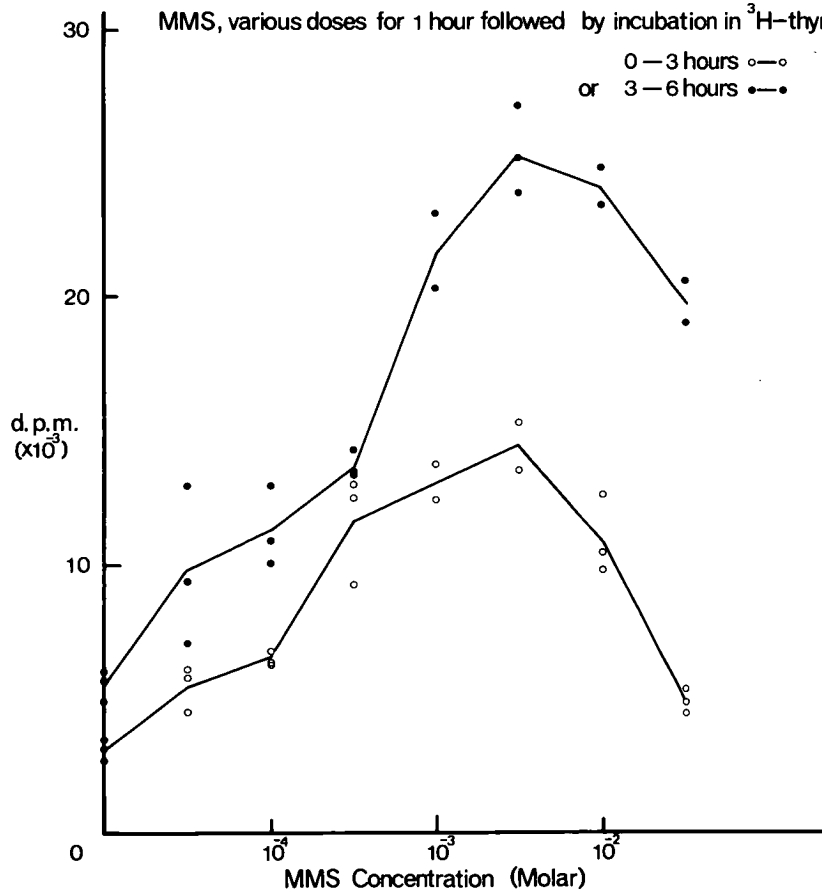


Fig. III 6 ^3H -thymidine uptake following incubation in various doses of HN2 (above) and MMS (below). -o- show uptake during the first 3 hours after treatment, three samples for each dose. Three further samples were incubated in "cold" medium for 3 hours and -●- shows uptake during the subsequent 3 hours.

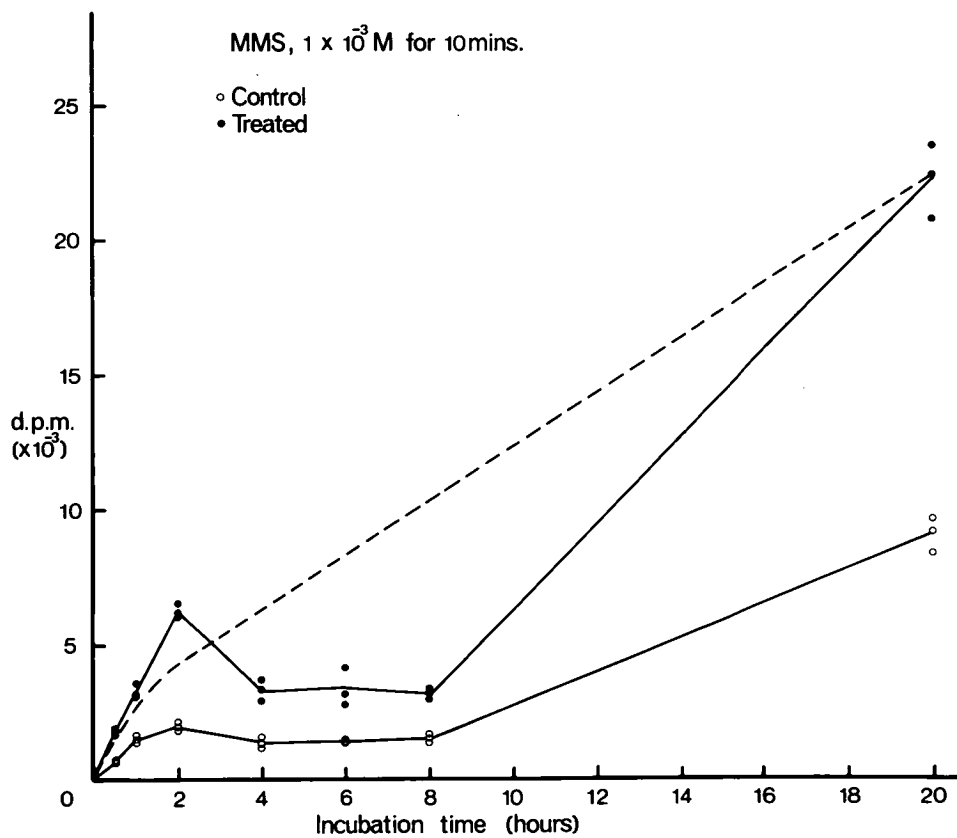
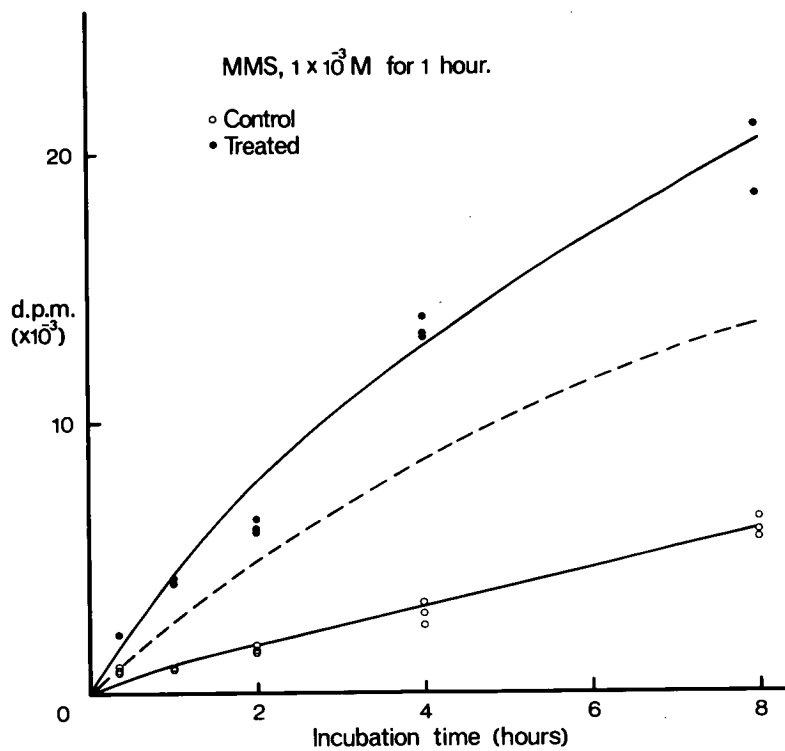


Fig. III 7 ^3H -thymidine uptake after incubation at 37°C in 10^{-3}M MMS; Three samples analysed at each time. In the upper fig. a one hour treatment was followed by continuous incubation in "hot" medium and in the lower fig. a ten minute treatment was followed by pulse labelling. In this case dpm values represent uptake over two hours prior to sampling (at 20 hours, from 8 to 20 hours), the cells first being incubated in "cold" medium. Net counts are shown as before and should be compared with fig. III 4.

there is an indication of a decrease in rate at 2 hours. A similar result was obtained at a dose level of $3 \times 10^{-3} \text{M}$ and also with a 1 hour incubation in 10^{-3}M MMS followed by continuous exposure to ^3H -thymidine labelling.

The incorporation at different dose levels was measured in the same way as in the HN2 experiments and was found to increase with increasing dose up to around $3 \times 10^{-3} \text{M}$ and then decline at higher concentrations. In contrast with HN2 there is a considerable amount of ^3H -thymidine taken up between three and six hours after exposure, in fact as much as that during the first three hours (see fig. III 6).

5. Initial rates of ^3H -thymidine incorporation

The rates of ^3H -thymidine uptake over the first 30 minutes post-treatment for a variety of doses of each mutagen were compared. The following doses were used: UV light: 0, 5, 10, 20, 50 and 150 ergs/mm^2 ; γ -rays: 0, 1, 2, 4, 10, and 30 k rads; HN2: 0, 10^{-5} , 2×10^{-5} , 4×10^{-5} , 1×10^{-4} and $3 \times 10^{-4} \text{M}$; MMS: 0, 2×10^{-4} , 4×10^{-4} , 8×10^{-4} , 2×10^{-3} and $6 \times 10^{-3} \text{M}$. ^3H -thymidine uptake after 0, 7, 15, 22 and 30 minutes post-treatment was measured for each mutagen and the rates of incorporation determined from the slopes of the lines of least squares fitted to each set of data. The reciprocal of the rate of incorporation for each dose was then plotted against the reciprocal of dose for each (fig. III 8) and in each case a straight line graph was obtained. The intercept of each extrapolated line onto the Y axis gives a measure of the rate on that line where the dose is infinitely large; these intercepts are shown in table III 2.

In the Michaelis Menten equation $V = \frac{E}{1 + K/S}$ where E = free enzyme concentration; S = concentration of radiation induced substrate

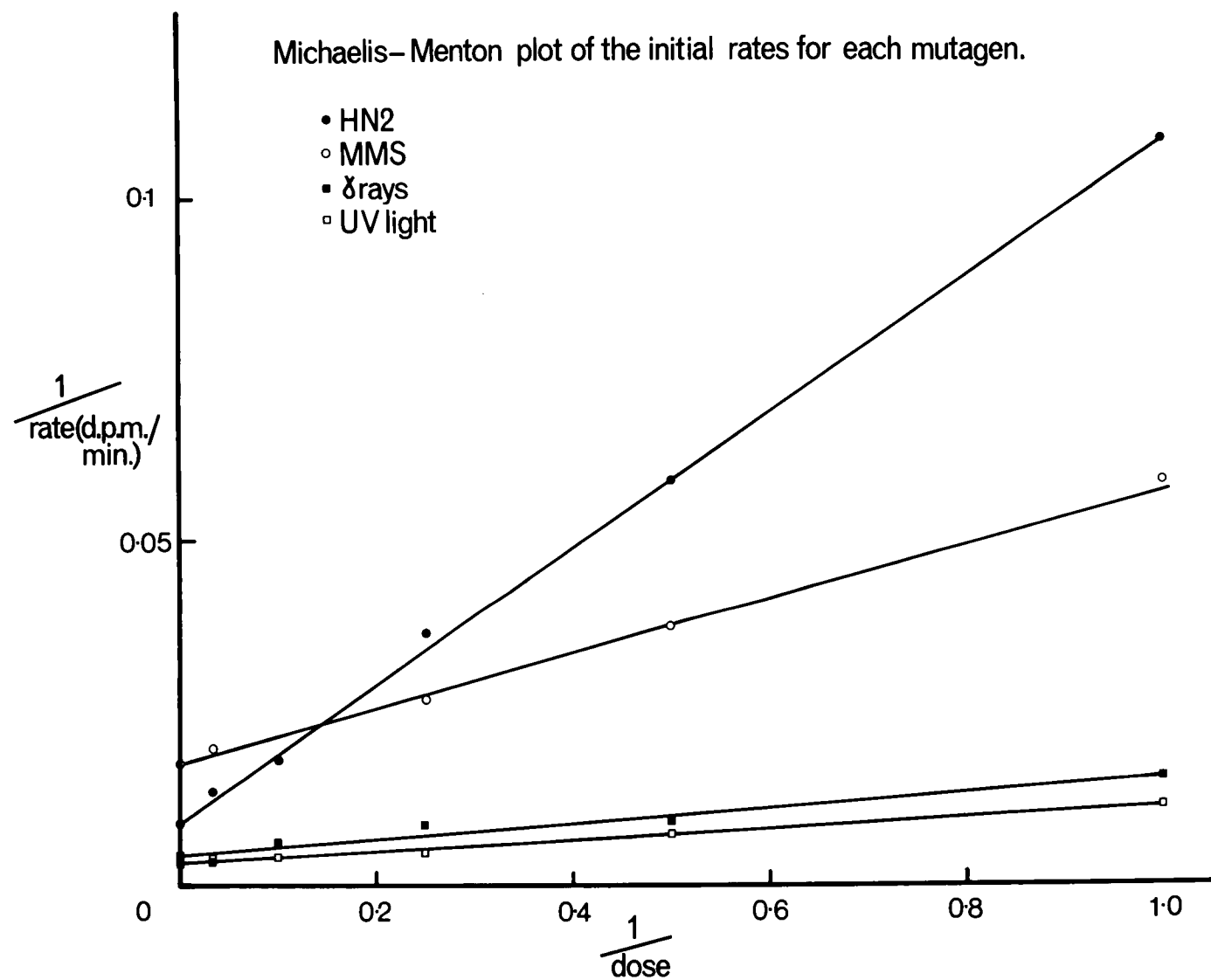


Fig. III 8 Michaelis Menten plot of initial rates of incorporation of ^3H -thymidine after each mutagen. Reciprocals of the initial rates are plotted against the reciprocal of dose. For each mutagen five doses in the ratio of 1:2:4:10:30 were used.

Table III 2 Intercepts of extrapolated Michaelis Menten plots
for UV light, γ -rays, HN2 and MMS.

Mutagen	Intercept	S.E.
U.V.	3.18	0.357
γ -rays	4.28	1.209
HN2	8.88	1.045
MMS	17.43	1.195

(lesions); V = rate; and K = rate constant. This equation can be rearranged to give: $\frac{E}{V} = 1 + \frac{K}{S}$

Since $\frac{1}{V}$ plotted against $\frac{1}{S}$ gives a straight line, the reaction is shown to be dependent on the free enzyme concentration (E). The fact that with the present data the rate at infinite dose (damage) differs for the different mutagens (except as between UV light and γ -rays) might therefore be taken to imply that different enzymes are involved in the rate limiting steps for the damage induced by these mutagens.

6. Effect of MMS and HN2 on Enzymes in vitro

It was suggested in the introduction to this chapter that chemical mutagens might react with cellular proteins and thereby interfere with the DNA repair mechanism. As it was not possible to estimate the effect of these mutagens on the repair enzymes, 2 enzymes whose activities could be estimated by simple assay systems in vitro, were incubated in various concentrations of HN2 and MMS in order to get some idea of the inhibitory action of these substances. The enzymes chosen were fumerase and lactic dehydrogenase. Following incubation in the mutagens, the enzymes were assayed as described in the Materials and Methods and the course of the reactions monitored optically. In table III 3 and III 4 the optical density of the fumerase assay mix at 5, 10, 15 and 20 minutes is shown following incubation of the enzyme in MMS and HN2 respectively. From graphs of these data the rates have been estimated (last column) and a plot of rate against mutagen concentration is shown in fig. III 9. It is evident that the mutagen has no effect on the activity of the enzyme until the concentration reaches $10^{-2}M$ in each case. For HN2 this results in a halving of the activity, an effect not evident in the MMS

Table III 3 The action of fumerase following incubation in MMS for one hour at 37°C estimated by increase in optical density.

MMS concentration (molar)	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
O.D. at 5 minutes	0.39	0.38	0.37	0.38	0.38	0.35
10 -	0.50	0.52	0.54	0.55	0.53	0.41
15 -	0.76	0.75	0.72	0.72	0.64	0.58
20 -	0.92	0.99	0.93	0.94	0.87	0.67
Rate (O.D./minute)	0.036	0.038	0.035	0.035	0.030	0.022

Table III 4 The action of fumerase following incubation in HN2 for one hour at 37°C estimated by increase in optical density.

HN2 concentration (molar)	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
O.D. at 5 minutes	0.58	0.54	0.49	0.54	0.51	0.43
10 -	0.93	0.94	0.79	0.82	0.83	0.60
15 -	1.20	1.13	1.09	1.16	1.09	0.76
20 -	1.47	1.37	1.32	1.40	1.41	0.93
Rate (O.D./minute)	0.056	0.050	0.055	0.057	0.057	0.034

Table III 5 The action of lactic dehydrogenase following incubation for one hour at 37°C in MMS or HN2 and estimated by drop in O.D. in unit time.

Concentration (molar)	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
MMS	0.22		0.18	0.18	0.20	0.20	0.06
HN2	0.23	0.23	0.22	0.24	0.24	0.17	

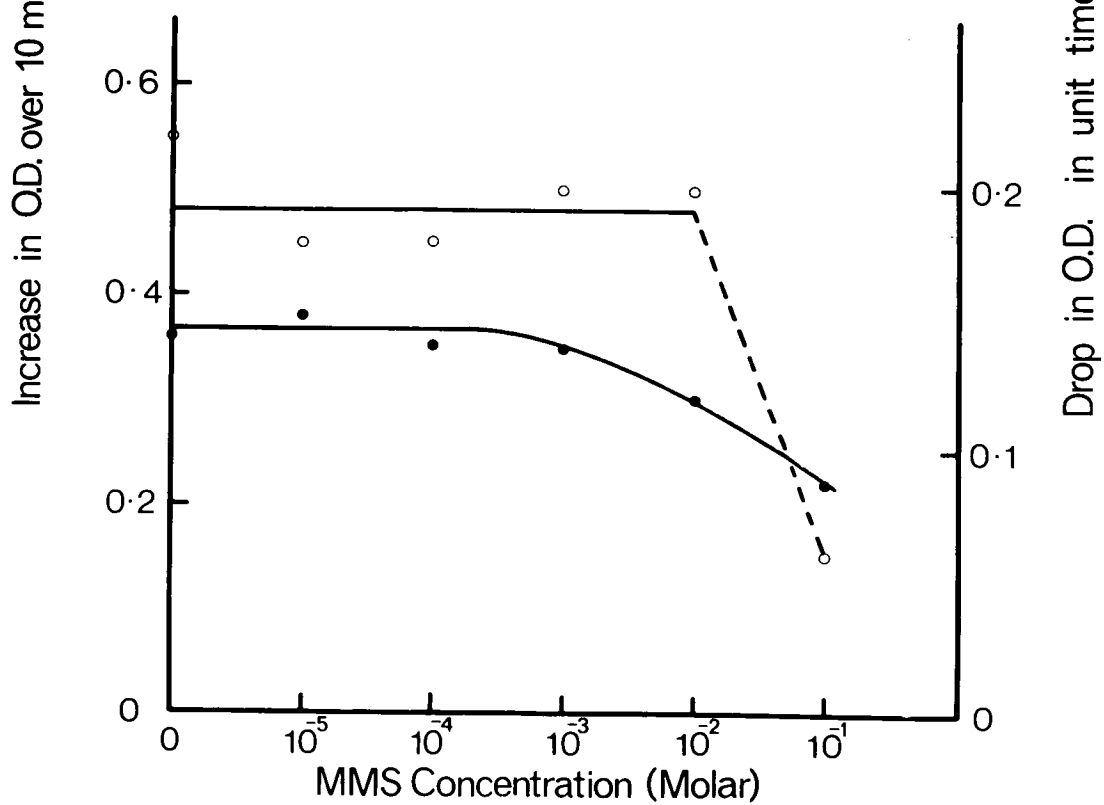
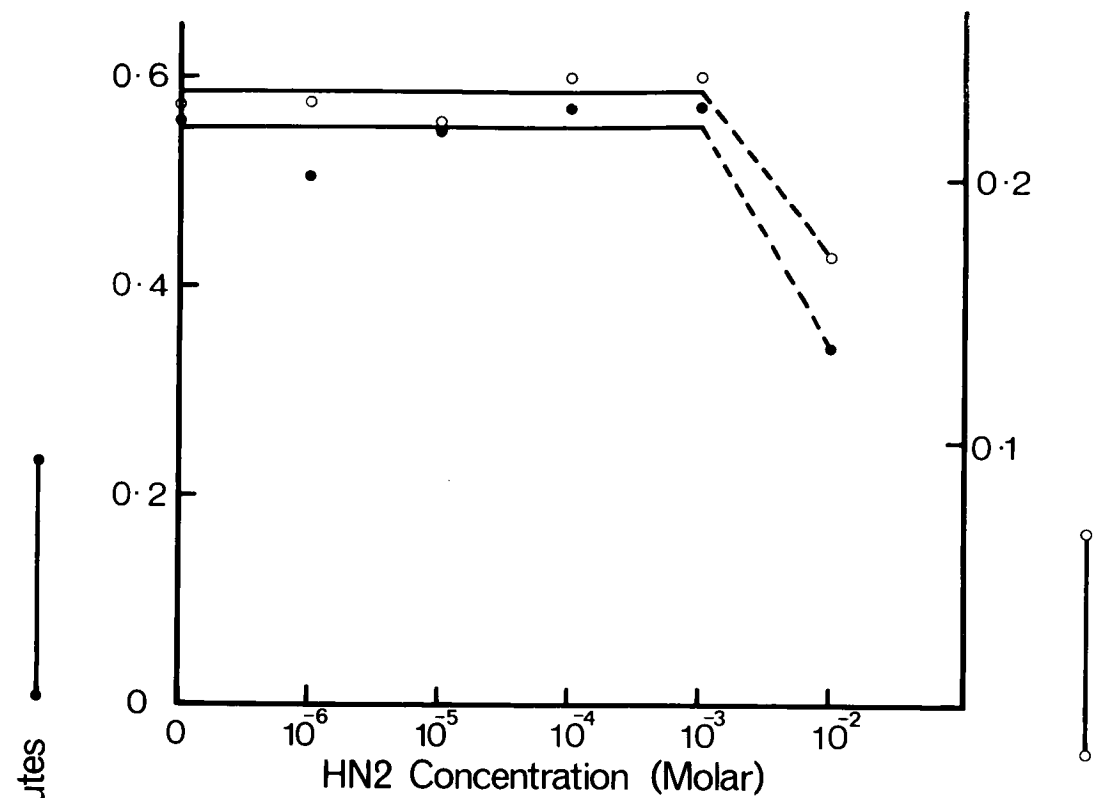


Fig. III 9 The effect of various concentrations of HN2 (above) and MMS (below) on the activity of fumerase and lactic dehydrogenase in vitro. For fumerase the increase in optical density during the first 10 minutes post-treatment was estimated (--●--) and for lactic dehydrogenase the initial rate of reaction was determined by optical scanning (--○--).

data until $10^{-1}M$ is reached.

The results for similar experiments with lactic dehydrogenase are shown in table III 5. The reaction was continuously scanned at 340m μ and the reduction in optical density in unit time estimated. This is plotted against concentration as before (fig. III 9) and shows a similar effect, the inhibitory action of the mutagens being marked at $10^{-2}M$ HN2 and $10^{-1}M$ MMS.

DISCUSSION

1. Dose Response

For each of the mutagens studied the response of the cells to a wide range of doses has been considered. The data for UV-irradiation covers the range 2 to 10,000 ergs/mm² and reveals a marked dose-dependence in the amount of synthesis up to 40 or 50 ergs/mm² followed by a plateau in the response until 5400 ergs/mm² is reached. Setlow et al (1963) showed for E.coli B/r that the number of dimers is proportional to dose and that $2.5 \times 10^{-4}\%$ of the thymine bases are dimerised per erg. If this can be extrapolated to mammalian cells it would indicate that at 40 ergs/mm² 0.01% of the thymines are dimerised and at this level the repair system is saturated.

This dose response curve agrees well with the rather more limited results independently obtained by Evans and Norman (1968) and Connor and Norman (1971) for lymphocytes and polymorphonuclear leucocytes irradiated with low doses of UV light. The low dose rate of γ -irradiation restricted the upper limit to 50,000 rads, but even so, there is evidence that the amount of induced synthesis is also dose-dependent resembling the early part of the UV dose response curve.

These results differ from those following treatment with a large range of concentrations of chemical mutagens. Here synthesis increases markedly with dose up to 10^{-4} M for HN2 and 3×10^{-3} M with MMS and then drops equally rapidly. These doses are considerably lower than those required to inhibit fumerase or lactic dehydrogenase in vitro (see fig. III 10) although it is difficult to relate this response to that of repair enzymes in vivo. Nevertheless the large plateau on the UV dose response curve would suggest an explanation of this kind in view of the more specific action of UV irradiation (see Introduction to this chapter).

This distinction between the dose response following irradiation and chemical treatments contrasts with differences in the temporal pattern of thymidine incorporation. Synthesis of DNA following exposure to UV-irradiation or to HN2 treatment is completed within 6 hours, whereas γ -ray or MMS-induced synthesis continues for at least 22 hours.

2. UV-irradiation and HN2 treatment

For a large range of doses the bulk of induced DNA synthesis after UV light or HN2 treatment is largely over within 3 hours and is virtually completed after six. This is in conflict with the results of Spiegler and Norman (1970) and the more recent data of Conner and Norman (1971) who claim that polymorphonuclear leucocytes (but not lymphocytes - Evans and Norman 1968a and b) continue synthesis for at least 8 hours post-irradiation in a manner resembling the γ -ray data. The present results are for a mixture of white blood cells and therefore precise estimations of rates, and changes in rates cannot be made. However at least half of the cells in the present work are

polymorphonuclear leucocytes and the amount of synthesis continuing after six hours is not comparable with that described by Norman et al. Autoradiographic studies (see fig. V 1) show that polymorphonuclear cells are labelled at least to the same extent as the lymphocytes. This in itself is an interesting finding (made independently of Norman et al) bearing in mind that these cells are differentiated end-cells with a very short life-span and no reproductive potential.

The different types of lesions induced in DNA by UV light and HN2 on the one hand and by γ -rays and MMS on the other may account for the different patterns of unscheduled DNA synthesis that are observed between these mutagens. Among the main and most important products in DNA exposed to UV-irradiation are the intrastrand pyrimidine dimers, and in particular the thymine dimer (see Introduction). Excision of dimers has been shown to occur in mammalian cells after exposure to UV (Cleaver and Trosko 1969; Regan et al 1968) and it is presumed that such excision is an early step in the repair process that results in the incorporation of labelled precursors into damaged DNA.

In the case of bifunctional alkylating agents, it is usually considered that the important lesion in DNA is the cross-linking via alkylation, of two guanines on opposite strands of the DNA (Brookes and Lawley 1961a; Goldacre et al 1949; Loveless and Stock, 1959). Both monoguanyl and diguanyl alkylation products are excised from the DNA of bacterial (Lawley and Brookes 1965; Papirmeister and Davidson 1964) and mammalian cells (Crathorn and Roberts 1966; Reid and Walker 1969) exposed to bifunctional alkylating agents; but there is some disagreement as to the relative preference for the elimination of diguanyl over monoguanyl products. Studies on HeLa cells



(Crathorn and Roberts 1968) suggest that there is random excision of alkylated products along both strands of the DNA, whereas in mouse L cells, Reid and Walker (1969) find that the loss of renaturable, i.e. cross-linked DNA, has a half life of 2 hours compared with 18 hours for the loss of total alkylated products, i.e. a preferential loss of diguanyl products. In E.coli B/r, Lawley and Brookes (1965) and Venitt (1968) report that some 50% of isotopically labelled HN2, and including all diguanyl products, are excised within 80 minutes following treatment.

Excised diguanyl alkylation products are often believed to be largely derived from interstrand cross links. Recent evidence, however, (Flamm et al 1970; Lawley et al 1969) has indicated that intrastrands cross links between adjacent guanines on a given DNA strand may be three or four times more frequent than interstrand cross links. Such intrastrand diguanyl products formed following HN2 treatment are formally comparable to the intrastrand pyrimidine dimers produced by UV. Both products are intrastrand alterations that will produce conformational changes within the DNA helix, and both might be expected to be subjected to the same mechanism of excision repair.

3. γ -Irradiation and NMS treatment

The γ -ray results are in accord with and extend previous results for induced unscheduled DNA synthesis following X-irradiation of human lymphocytes (Spiegler and Norman 1969) and mouse L cells (Hill 1967). Spiegler and Norman (1970) have stressed the likely involvement of two reactions with different rates, a fast reaction which is completed in the first hour following exposure and a slow reaction that continues for at least 7 hours. It was suggested that the initial fast reaction

might involve the incorporation of only one or two nucleotides per lesion, agreeing well with the estimations made here. Little DNA degradation accompanies this synthesis (Painter 1968; Little 1968; Hill 1969) unlike that following UV irradiation and it is tempting to conclude that the fast reaction process observed after exposure to ionising radiations might be involved in the rejoining of single strand breaks in the DNA. Molecular weight studies indicate that this rejoining occurs largely during the first 60 minutes post-irradiation (Humphrey et al 1968; Kleijer et al 1970; Lett et al 1967; McGrath and Williams 1966; Ormerod and Stevens 1971) although these studies do not take into account the reduction in molecular weights incurred during the isolation of the DNA.

The possible association between this fast reaction and strand rejoining is strengthened by the recent work of Donlon and Norman (1971) who have compared the kinetics of rejoining of single strand breaks in the DNA of X-irradiated lymphocytes with the kinetics of unscheduled DNA synthesis in these cells. Their results indicate that the repair of single strand breaks and the stimulated incorporation of nucleotides share the same rate-limiting enzymatic step. The fact that the two reactions have differing dose-responses is also of interest particularly as that for the initial fast reaction plateaus at approximately the same dose level as that for the rejoining of single-strand breaks (Donlon and Norman 1971).

If the fast reaction can be accounted for in this way, what is the significance of the slow prolonged synthesis, unless of course some of the breaks develop sometime after the absorption of the energy? Sawado and Okado (1970) have shown that actinomycin D

completely suppresses the repair of sub-lethal damage in L5178Y (murine lymphoma) cells as measured by cell survival, but has no effect on the rejoining of breaks. It is therefore possible that this synthesis is associated with the repair of damage other than single-strand breakage. Base damage and cross-linking of DNA to protein is also known to be a consequence of X-irradiation (see Introduction). Errors could also arise during the rapid rejoining of broken strands resulting in the elimination of bases or even cross-linking of two DNA strands. However this DNA synthesis is of increasing importance at higher doses and these data show that it may account for the majority of label incorporated into the DNA. As the number of strand breaks would also be expected to increase by the same amount, it may represent a random addition of label onto the broken ends of the DNA as suggested by Ayad et al (1969) and Fox and Fox (1969) who were unable to relate non-semiconservative synthesis to strand rejoining in lymphoma cells exposed to MMS. An attempt to elucidate this aspect has been made in the next chapter.

Exposure to MMS is known to result in DNA strand breakage (Strauss and Wahl 1964; Strauss and Hill 1970) and again molecular weight studies have shown that reconstruction of the DNA occurs (Fox and Fox 1970). It is therefore not surprising that the pattern of induced DNA synthesis following MMS resembles the γ -ray data, showing a two-phased reaction and lasting for at least six hours at all doses. However strand breakage following MMS treatment is a secondary reaction resulting from hydrolysis of the alkylated purines to give apurinic sites, and this would account for a less obvious initial fast reaction compared to that obtained with X- or γ -rays.

In conclusion, then, it appears that for the four mutagens used in this study, two types of reaction are initiated in the DNA. These appear to be related to the types of damage induced; unscheduled DNA synthesis lasting 4 to 6 hours following UV light and HN2 which are known to produce conformational changes in the DNA helix, and an extended synthesis following mutagens in which the primary lesion is thought to be strand breakage. The results in Fig. III 8 and Table III 2 do however suggest that different rate-limiting steps are involved in the reaction following each of the mutagens. Despite this a parallel pattern of response to cell killing by these agents has been described for microorganisms (see Chapter VI), again implicating common mechanisms on the one hand for UV and HN2 damage, and on the other for γ -ray and MMS-induced damage.

IV. CHARACTERISATION OF THE INDUCED SYNTHESIS

INTRODUCTION

It was established in the last chapter that unlike the DNA synthesis induced by exposure to UV or HN2, which was virtually completed in the first 4 to 6 hours following treatment, that following γ -irradiation and MMS treatment was prolonged and extended over many hours. Experiments by Roberts et al (1971b) on "repair replication" in HeLa cells exposed to sulphur mustard show a somewhat extended, but very similar temporal pattern to these for unscheduled DNA synthesis following HN2. Such a similarity is of course to be expected if unscheduled DNA synthesis and "repair replication" following alkylating agents are a reflection of the same "repair" process.

In the case of γ -irradiation (and MMS) the situation is by no means as clear. In relation to the present γ -ray findings somewhat similar, but rather more limited, results for unscheduled DNA synthesis have been obtained by Spiegler and Norman (1969) and Hill (1967) for X-irradiated lymphocytes and mouse L cells. However studies on "repair replication" following X-irradiation of HeLa (Brent and Wheatley 1971) and lymphoma cells (Fox et al 1970) suggest that this type of synthesis lasts for 1-2 hours following exposure, a similar duration to that of the initial fast reaction described in the experiments in which unscheduled DNA synthesis was measured. This fast reaction has been correlated with the rejoining of single-strand breaks involving the insertion of 1-2 nucleotides per break (see Chapter III), an incorporation which would manifest itself as "repair replication". This rejoining of single-strand breaks is completed in

1-2 hours (Humphrey et al 1968; Kleijer et al 1970; Lett et al 1967; McGrath and Williams 1966; Ormerod and Stevens 1971). However between 2 and 24 hours post-irradiation as much label is incorporated as in this initial 2-hour period, and the problem is therefore what this incorporation represents.

Several possibilities may be proposed to account for the continued incorporation of ^3H -thymidine as a result of exposure to γ -rays or MMS. It has already been suggested that it could be associated with the repair of damage other than single-strand breakage e.g. damage to individual bases. However, this situation might in formal terms be comparable to that found following UV-irradiation and prolonged "repair replication" would not be expected. Experiments which demonstrate "repair replication" depend on the fact that the newly-synthesised regions are small relative to the size of the DNA fragments on the gradient, so that an apparent absence of "repair replication" could be due to the replacement of large regions of the DNA, degradation and resynthesis continuing well beyond the damaged bases. If this were so, then increasing post-irradiation incubation times would result in a gradual shift in density of the newly-synthesised DNA towards that of DNA fully-labelled with the density marker (BUdR-substituted DNA if BUdR used during "repair", or towards normal density DNA if ^3H -thymidine used in conjunction with a BUdR-prelabel). The possibility that large regions of DNA are being degraded and resynthesised is somewhat unlikely, though, in view of the many unsuccessful attempts to demonstrate DNA degradation following moderate doses of X-rays (Little 1968; Painter 1968; Hill 1969).

Another possibility, already suggested by Ayad et al (1969) to

explain the MMS-induced synthesis in lymphoma cells, is the incorporation of nucleotides onto broken ends of the DNA molecule.

In the case of the γ -ray data, the rate of synthesis 2 hours after irradiation is directly proportional to the dose and continues at a constant rate; not incompatible with this explanation. Also, on CsCl gradients the newly-synthesised regions would have a greater chance of becoming detached from the parental strand as they would be attached at one end only.

With these factors in mind, the experiments in this chapter were designed to determine whether all of the extensive synthesis following MMS and γ -rays was true "repair". As explained earlier, the absence of "repair replication" (Rasmussen and Painter 1966) at long incubation times post-treatment could be due to the increase in length of each newly-synthesised region so that their density changed; or to some other phenomenon not related to recovery mechanisms, such as end-addition of nucleotides. The latter was investigated using exonuclease degradation of double-labelled ($^{14}\text{C}/^3\text{H}$) samples.

For the density label experiments, following mutagen treatment, the cells were incubated in BUdR and ^3H -CdR, or ^3H -thymidine in cells prelabelled with BUdR, and the density of the ^3H -labelled DNA relative to the main band was sought. There is already evidence to suggest that large, fully-substituted, pieces of DNA are produced following exposure to X-rays (Painter and Young 1972) and an attempt to demonstrate an increase in the size of these regions with incubation time was made. In order for these results to be comparable with those described in Chapter III, unstimulated (G_1) cells were used in many experiments and thus the first type of experiment involving BUdR

uptake during "repair" employed. Where a BUdR prelabel was used, the cells had to be stimulated to undergo "normal" semi-conservative DNA synthesis, so that the mutagen treatment was applied to a mixed cell population containing cells in all stages of the cell cycle. This latter situation is more comparable with that obtaining in work already published for established fibroblast and lymphoma cell lines, and a comparison of the two situations was thought to be of value.

The second set of experiments was designed to investigate the possibility of end-addition of nucleotides onto broken DNA strands. The DNA was pre-labelled with ^{14}C -thymidine and following mutagen treatment the cells were incubated in medium containing ^3H -thymidine. Following isolation and denaturation of the DNA its pattern of degradation by exonuclease was followed; the loss of ^{14}C giving an estimate of the overall rate of degradation and the rate of loss of ^3H relative to this, giving an indication of the distribution of the newly-synthesised regions. It was postulated that ^3H -thymidine incorporated in a short pulse (5 minutes) during S synthesis (in the absence of mutagen) would be preferentially degraded or untouched (depending on the direction of attack of the enzyme) in a manner similar to that due to end-addition, and this was used as a control for the system. ^3H -thymidine incorporated as a result of true repair (assumed to be the case following UV irradiation) was expected to result in the loss of both isotopes at the same rate.

RESULTS

The incorporation of ^3H -nucleotides into DNA following mutagen treatment was studied in two ways. The cells were either pre-labelled with ^{14}C -BUdR, incubated for 2 hours in the absence of label,

treated with mutagen and incubated for various times in medium containing ^3H -thymidine, either continuously or as a "pulse" label (see chapter II). The DNA was then isolated on alkaline Cs_2SO_4 gradients and the incorporation of ^3H into the heavy (BUdR-labelled) strand taken as a measure of the amount of "repair replication".

A second method, and the only means of studying non-stimulated cells, was to follow the incorporation of BUdR into normal-density DNA. A ^{14}C -thymidine pre-label or ^{14}C -labelled DNA was used as a marker to identify the position of normal density DNA and ^3H -CdR was used in conjunction with the BUdR (as ^3H -CdR was of greater specific activity than ^3H -BUdR and resulted in a much larger incorporation of ^3H) to show the position of the newly-synthesised regions. In the case of stimulated cells, a 2 hour incubation in BUdR prior to mutagen treatment was employed to prevent continued S synthesis resulting in anomolous results as in the case of cells prelabelled with BUdR (see Chapter II). Following post-mutagen incubation, the DNA was isolated on alkaline CsCl gradients.

1. UV light

The induction of DNA synthesis by UV light is shown in fig. IV 1 and IV 2 using a "pulse" label. In fig. IV 1 the cells were prelabelled for the first generation with ^{14}C -CdR, followed by one generation in ^{14}C -BUdR. This resulted in both strands of the DNA being labelled with ^{14}C and in addition one of these (in which BUdR was incorporated) was also of increased buoyant density. After 2 hours in "cold" medium (devoid of isotope or BUdR) and $\frac{1}{2}$ hour in 10^{-3}M hydroxyurea, they were suspended in saline and irradiated with 700 ergs/mm^2 . The 4 samples were incubated respectively for 0-2,

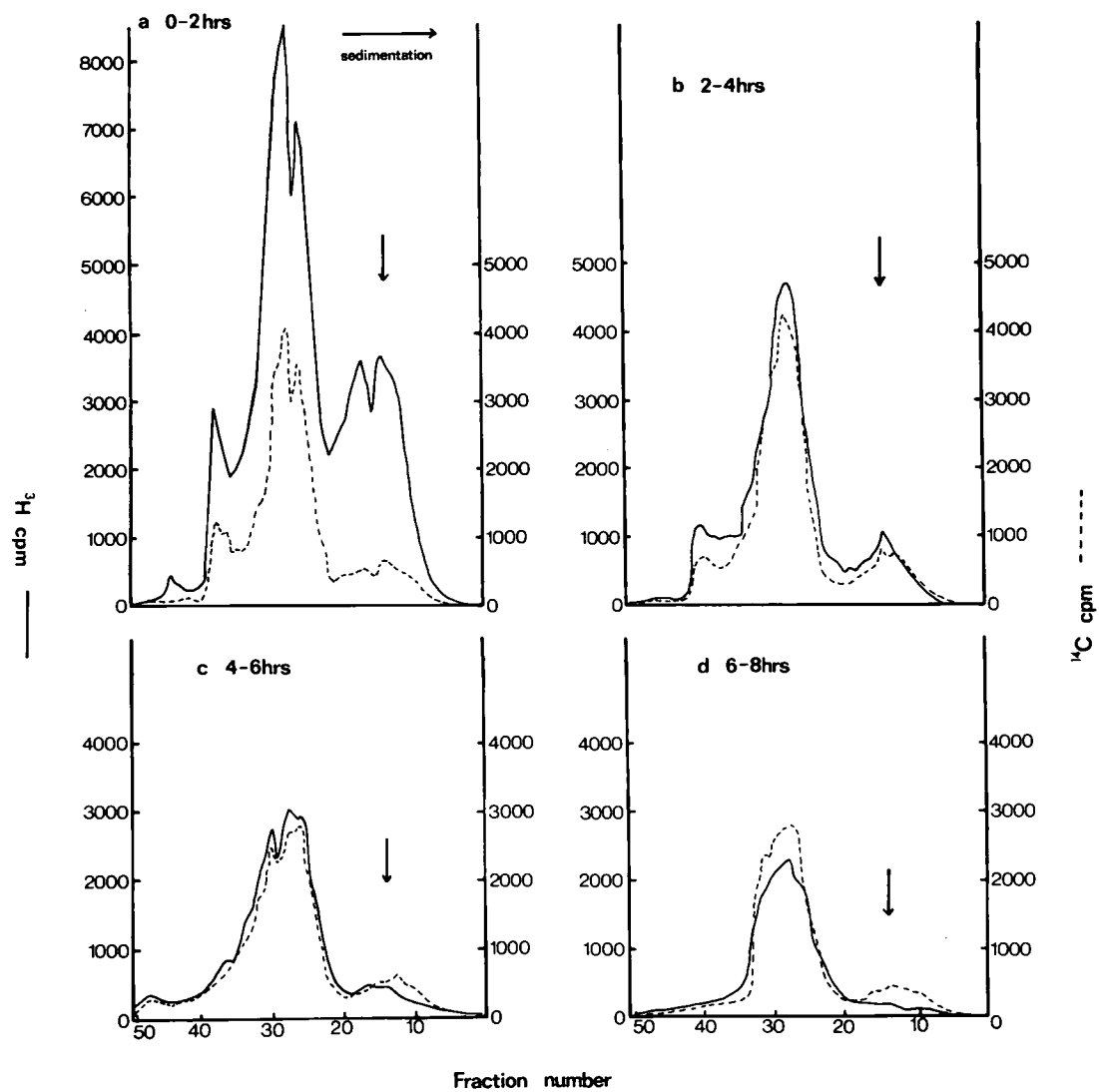


Fig. IV 1 Alkaline Cs_2SO_4 gradient profiles of DNA from cells prelabelled with ^{14}C -CdR and ^{14}C -BUdR (one generation each) and irradiated with 700 ergs/mm^2 UV light.

- a) Incubated 0-2 hours in ^3H -thymidine post-irradiation
- b) Incubated 0-2 hours in "cold" medium and 2-4 hours in ^3H -thymidine
- c) Incubated 2-4 hours in "cold" medium and 4-6 hours in ^3H -thymidine
- d) Incubated 4-6 hours in "cold" medium and 6-8 hours in ^3H -thymidine

The heavy (BUdR-labelled) DNA is arrowed; note the reduced rate of incorporation of label with time post-irradiation.

2-4, 4-6 and 6-8 hours post-irradiation in ^3H -thymidine following incubation in "cold" medium where appropriate. It is evident that ^3H -thymidine has been incorporated into both the heavy (BUdR labelled) and light strands of the DNA and that this incorporation diminishes with time post-irradiation. The reduction in incorporation with time after irradiation is most noticeable in the case of the heavy strand. Some of the incorporation into light strands could be due to residual S synthesis whereas all of the incorporation into the BUdR-labelled strand must represent "repair replication" and the process appears to be completed within 6 hours post-irradiation. It is impossible to tell from this experiment the relative amounts of semi-conservative and repair synthesis in the light strand, but it has already been shown that the BUdR-labelled strand is preferentially damaged (and repaired) following UV irradiation (Cleaver 1968a) and it is likely that most of the synthesis in the light strand at 6-8 hours is due to continued S synthesis.

When ^3H -CdR and BUdR incorporation into the DNA of UV-irradiated non-stimulated cells is studied (fig. IV 2) a similar result is obtained. The incorporation at 2-4 hours is only one third of that at 0-2 hours post-irradiation and by 4-6 hours it is almost the same as the control level. A ^{14}C marker DNA has been added to the gradients to show the position of normal density DNA and all the ^3H counts coincide with it. Experiments with stimulated cells have shown that S synthesis during a 2-hour incubation period in the presence of BUdR and ^3H -CdR results in ^3H counts at a density greater than that of normal DNA and therefore all the synthesis shown in fig. IV 2 must be due to "repair replication".

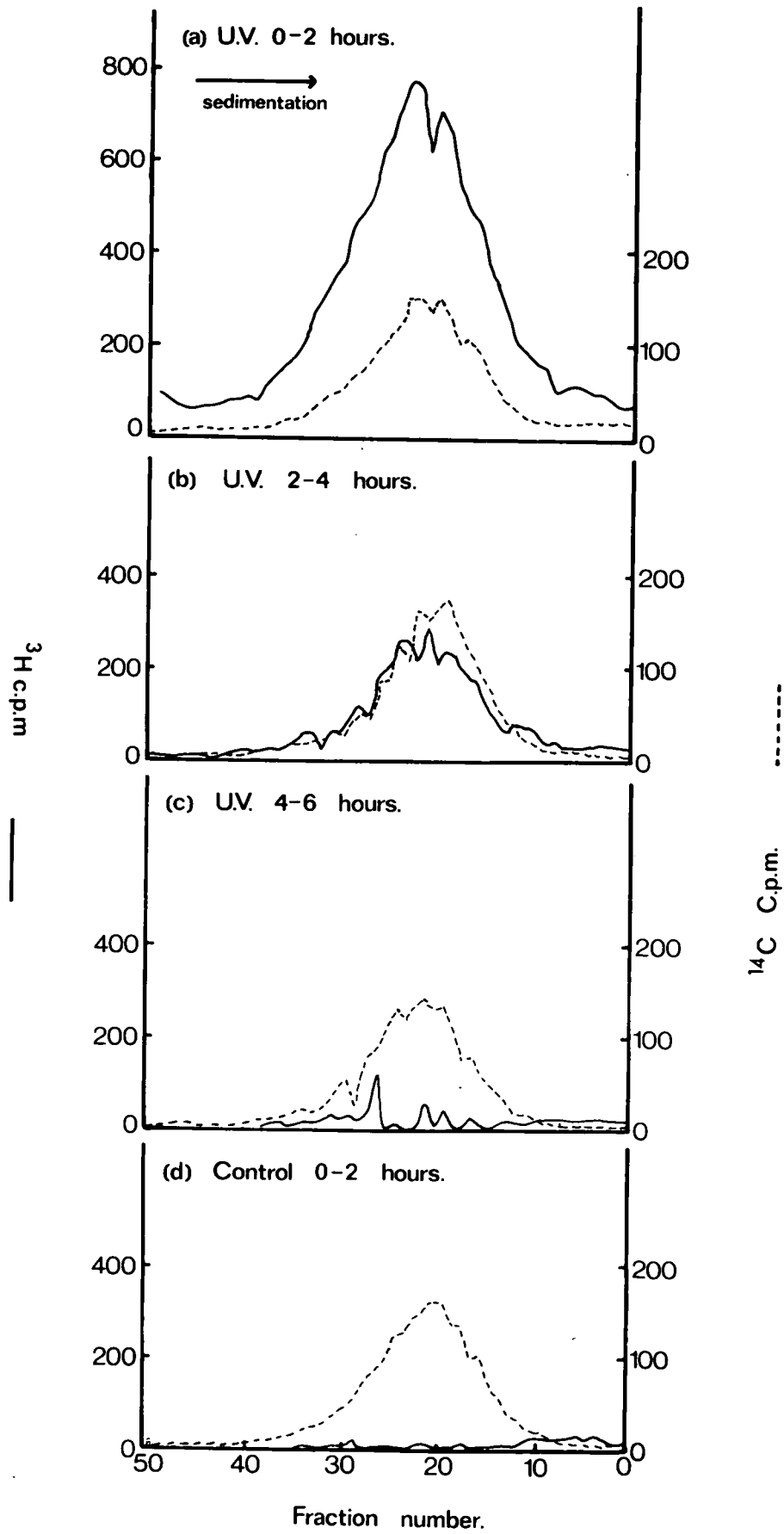


Fig. IV 2 Alkaline CsCl gradient profiles of DNA from non-stimulated cells exposed to 125 ergs/mm^2 UV light and incubated in BUdR and $^3\text{H-CdR}$. A ^{14}C -marker DNA was added to the gradients to denote the position of normal-density DNA.

- a) Incubated 0-2 hours in BUdR and $^3\text{H-CdR}$ post-irradiation
- b) Incubated 0-2 hours in BUdR and 2-4 hours in BUdR and $^3\text{H-CdR}$
- c) Incubated 2-4 hours in BUdR and 4-6 hours in BUdR and $^3\text{H-CdR}$
- d) Unirradiated control incubated for 2 hours in BUdR and $^3\text{H-CdR}$

Note the reduction in the rate of incorporation of label with time post-irradiation as in the stimulated cells (see Fig. IV 1).

2. MMS and γ -rays on Stimulated Cells

In fig. IV 5 and IV 6 the incorporation of ^3H -thymidine into hybrid (BUdR-labelled) DNA following MMS and γ -rays has been compared with that following UV-irradiation. Previous experiments with MMS and γ -rays had shown a much smaller incorporation of ^3H -thymidine into BUdR-labelled DNA as compared with that due to UV irradiation and a much greater amount of S synthesis was still evident despite the mutagen treatment and the presence of hydroxyurea. In fig. IV 3 the effect of various concentrations of hydroxyurea on both S synthesis and UV-induced synthesis are shown. The latter is unaffected by all concentrations of hydroxyurea, even up to $5 \times 10^{-1}\text{M}$. However S synthesis is increasingly depressed up to 10^{-3}M , above which there is no further effect, 4% of the synthesis remaining completely insensitive to the drug at all concentrations. The unstimulated, unirradiated control cells also have a small amount of spontaneous synthesis, only a percentage of which is sensitive to hydroxyurea.

In order to study repair replication in the absence of S synthesis hybrid DNA was isolated on neutral CsCl and rebanded by spinning in alkaline Cs_2SO_4 . In this was it was possible to study the incorporation of ^3H -thymidine into the light and heavy (BUdR) strands present in known (equal) relative proportions. It was also possible to compare template and newly-synthesised DNA by treating the cells either immediately after BUdR labelling or one generation later. In the case of the former, the BUdR-labelled strand is newly-synthesised and thus the light strand is acting as a template and will contain very little ^3H -thymidine due to S synthesis.

The experimental details are summarised in fig. IV 4. The cells

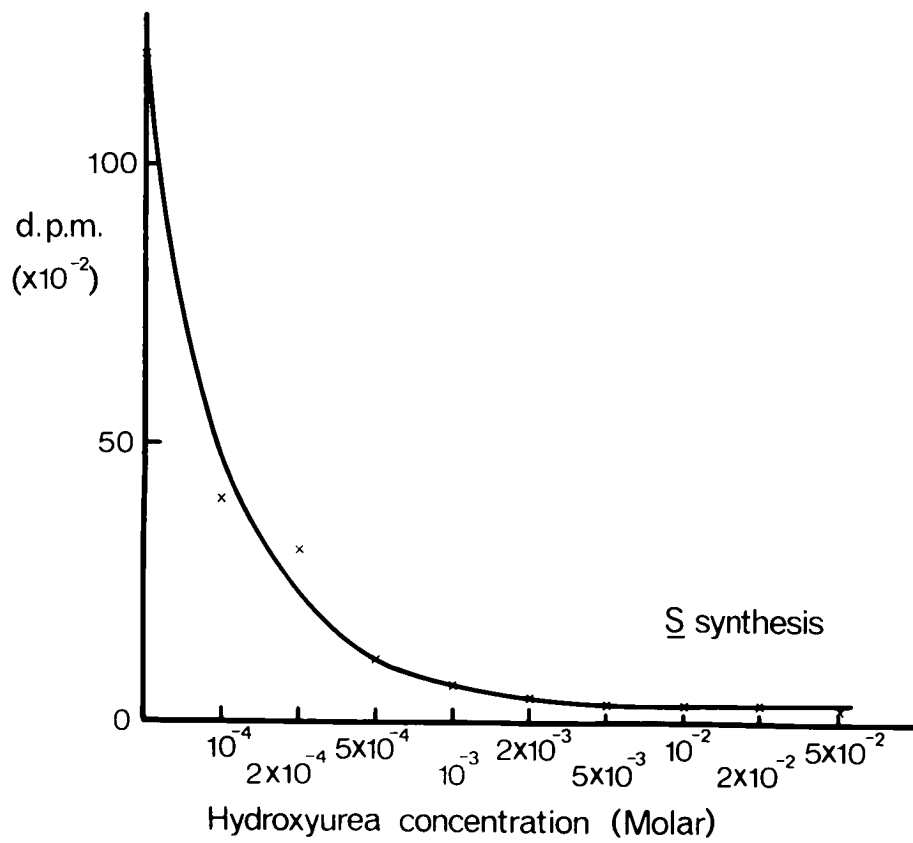
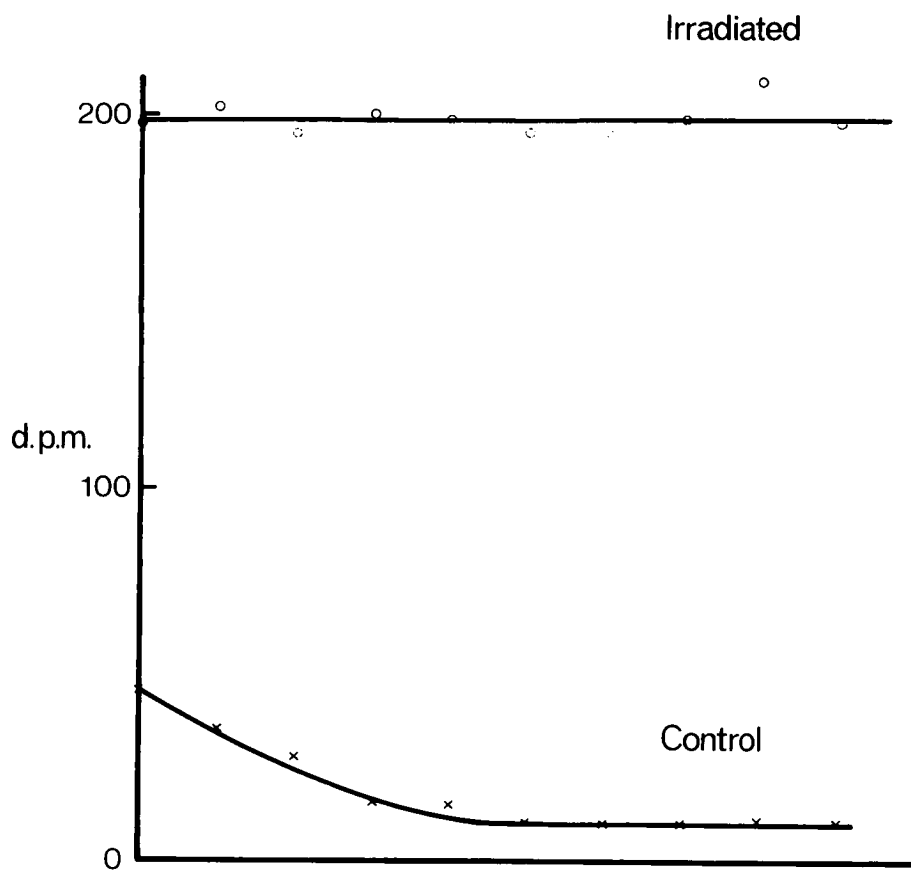


Fig. IV 3 The effect of various concentrations of hydroxyurea on unscheduled DNA synthesis (upper fig.), and S synthesis (lower fig.) in PHA-stimulated lymphocytes, estimated as ^3H -thymidine incorporation into TCA-insoluble material. In the former G_1 lymphocytes were untreated (control) or irradiated with 700 ergs/mm^2 UV light prior to incubation in hydroxyurea and ^3H -thymidine for 3 hours.

were incubated in ^{14}C -BUdR for one generation and then incubated in the absence of label for one hour or for 24 hours. After $\frac{1}{2}$ hour in hydroxyurea, half of each sample was irradiated with 5000 rads of γ -rays and incubated in medium containing ^3H -thymidine for 6 hours. The latter is incorporated either for continued S synthesis or as a result of "repair replication". The DNA was isolated on neutral CsCl gradients and fig. IV 4 shows the types of molecule expected, assuming that a certain amount of shearing of the DNA has occurred. The actual density profiles obtained are shown in fig. IV 5. The initial gradients are shown in the insets together with the fractions selected for rebanding in alkaline Cs_2SO_4 . In the top figs. the BUdR strand is newly-synthesised and the unirradiated control shows that there is no "repair replication" in it. In addition very few counts (due to S synthesis) are present in the light (template) strand consistent with the pattern predicted in fig. IV 4. After γ -irradiation the number of counts in this light strand is comparable with that in the control (c.f. ^{14}C : ^3H counts) but in addition there is an appreciable amount of label in the heavy strand due to "repair replication". This is also evident when the BUdR strand is the template (lower fig.) despite the large number of counts now present in the light (newly-synthesised) strand in both the control and irradiated cells.

Fig. IV 6 shows a similar experiment for cells treated with 700 ergs/mm² UV light or 10^{-3}M MMS. ^{14}C -CdR was present prior to the ^{14}C -BUdR in order to label both strands of the DNA with ^{14}C . The MMS-treated cells show a similar pattern of uptake to the γ -ray data, although the amount of incorporation into the light strand

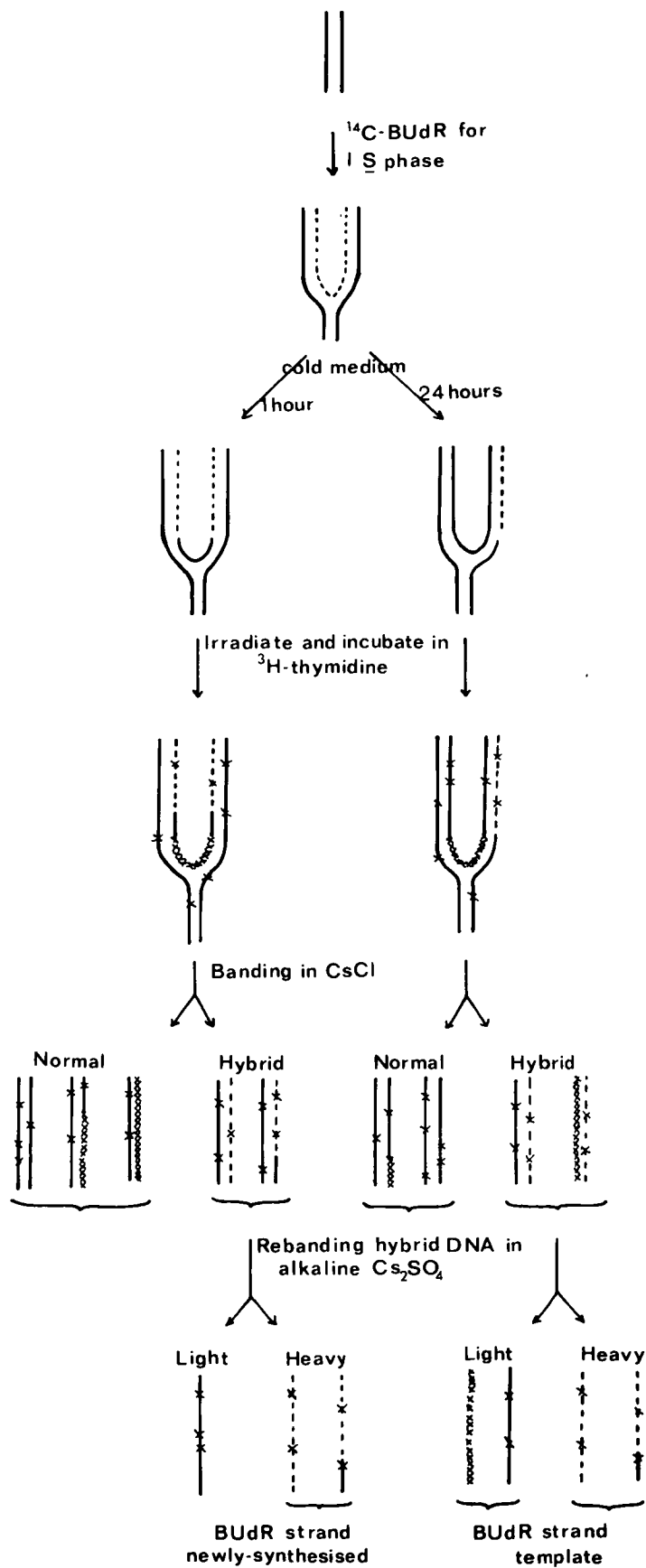


Fig. IV 4 A summary of the experiment to compare "repair replication" in template and newly-synthesised DNA. The predicted composition of DNA from cells labelled with ^{14}C -BUdR for one generation irradiated and incubated in ^3H -thymidine is shown. Its density profile on neutral CsCl and subsequent rebanding of the hybrid DNA on alkaline Cs_2SO_4 is shown in Fig. IV 5.

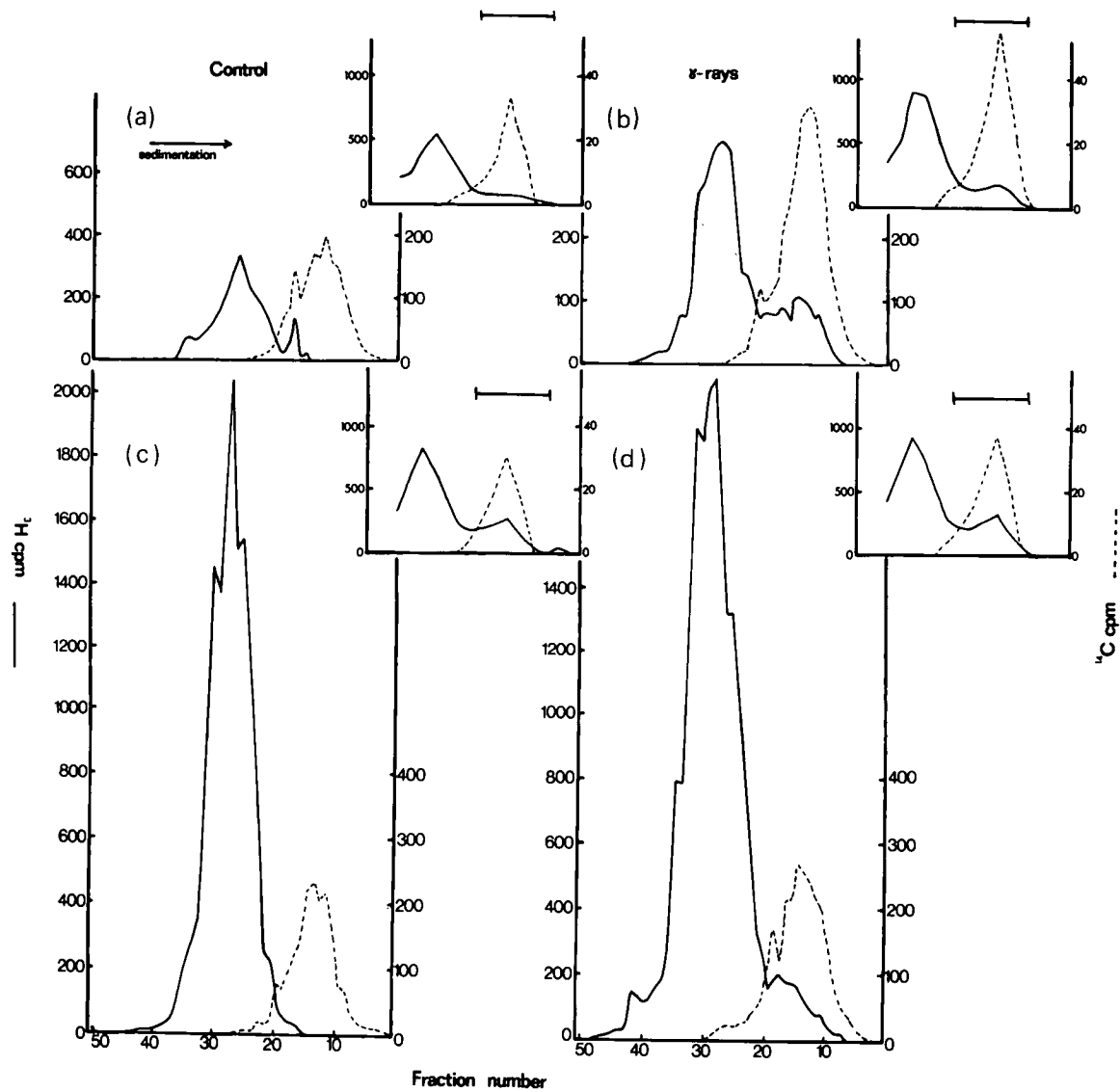


Fig. IV 5 ^3H -thymidine uptake by newly-synthesised and template DNA. The cells were labelled for one generation in ^{14}C -BUdR and half irradiated with 5000 rads of γ -irradiation after one hour (upper fig.) or 24 hours (lower fig.) incubation in "cold" medium. They were then incubated in ^3H -thymidine for 6 hours and the DNA isolated on neutral CsCl gradients (inset). — denotes regions for rebinding in alkaline Cs_2SO_4 gradients. A summary of the experimental details is shown in Fig. IV 4 together with the predicted composition of the DNA in each case. Note the similarity in the amount of ^3H in the BUdR-labelled strand (due to "repair replication") whether it is the template or newly-synthesised strand, but the increased amount of ^3H in the light strand when it is newly-synthesised (lower figs.).

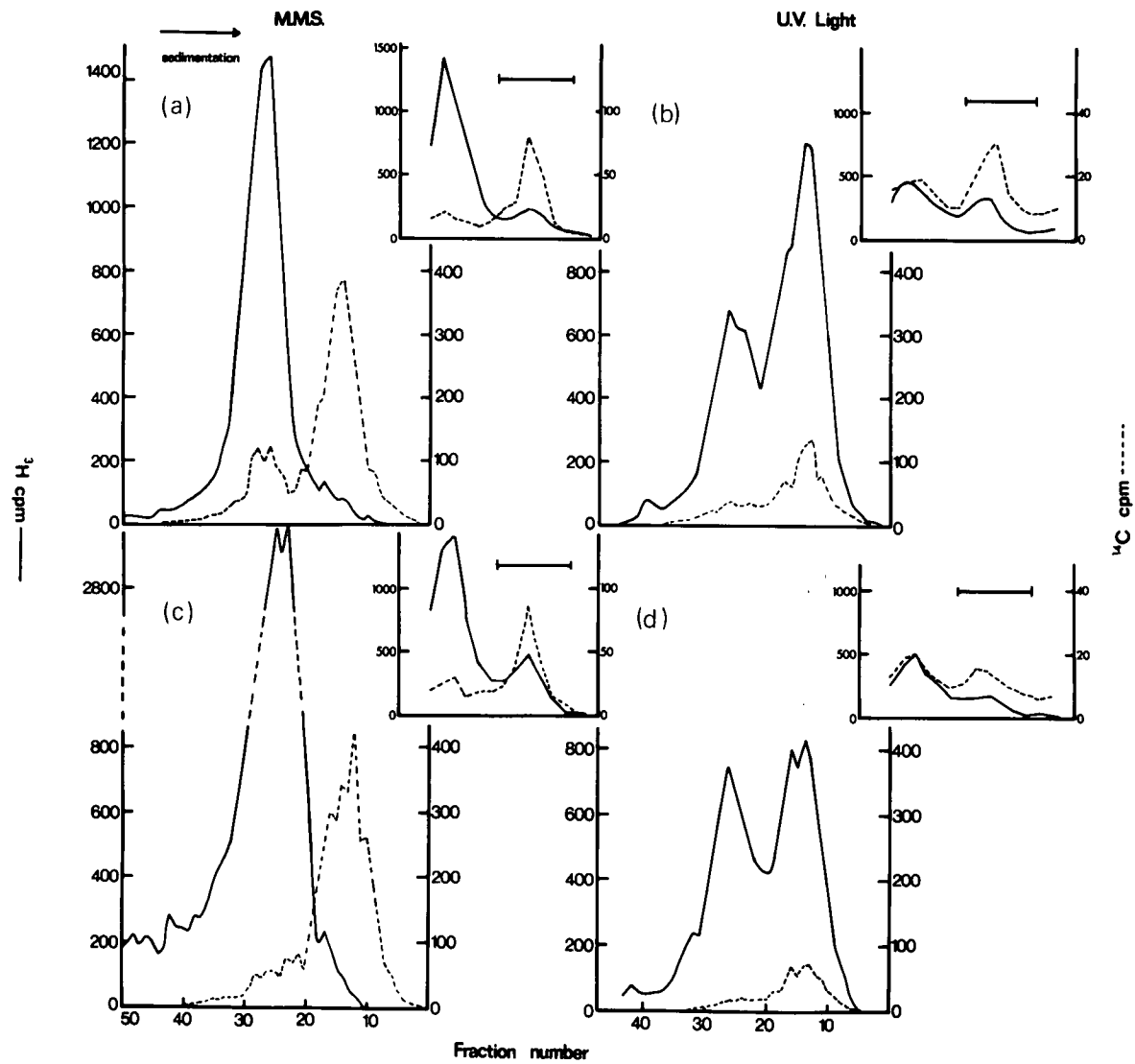


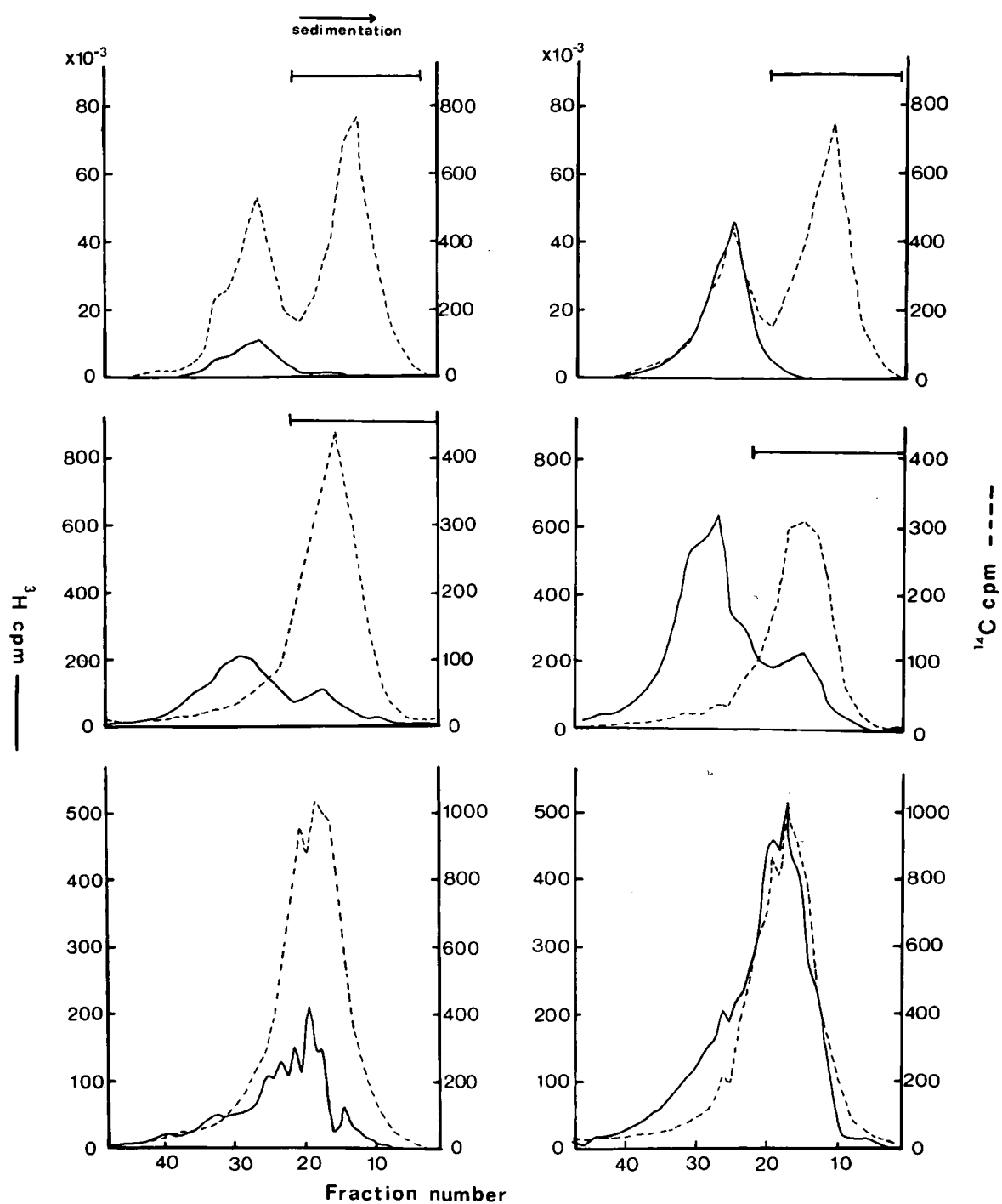
Fig. IV 6 ^3H -thymidine uptake by newly-synthesised and template DNA. The cells were labelled for one generation in ^{14}C -CdR and a second generation in ^{14}C -BUdR. After one hour (upper fig.) or 24 hours (lower fig.) incubation in "cold" medium they were either UV-irradiated or treated with 10^{-3}M MMS for 1 hour. Following incubation in ^3H -thymidine for 6 hours the DNA was isolated on neutral CsCl gradients (inset) and the fractions marked (a) and (b) rebanded in alkaline Cs_2SO_4 . These results should be compared with those in Fig. IV 5; note the marked depression of S synthesis by UV-irradiation and the preferential labelling ("repair") of the BUdR-labelled DNA.

is somewhat greater. The UV results, however, differ considerably. In the upper fig. (where S synthesis is negligible) the BUdR strand shows more "repair replication", consistent with previous data (Cleaver 1968a) and by comparing $^3\text{H}:^{14}\text{C}$ ratios in the two figs., a similar amount of repair in this strand is evident when it is acting as the template. The slightly greater proportion of counts in the light strand in the lower fig. is presumably due to S synthesis. The latter is however very appreciably depressed by the UV irradiation as can be seen by comparing the amount of ^3H in the light DNA on the initial neutral gradients.

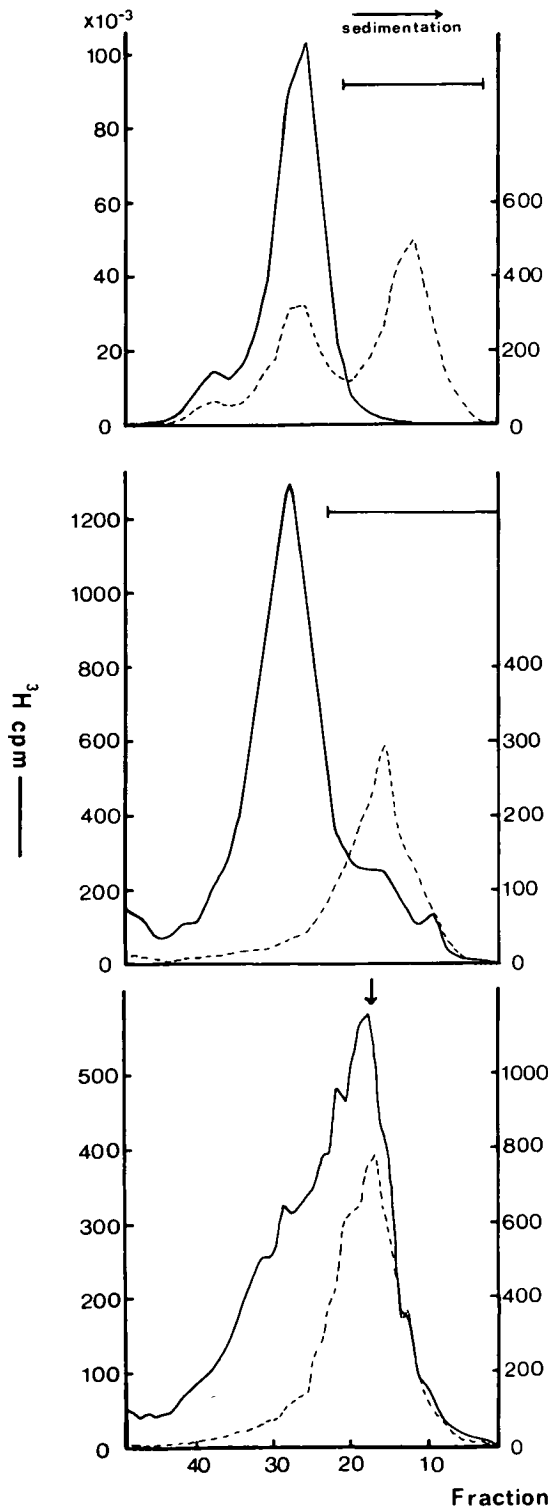
The amount of "repair replication" over longer time periods post-mutagen treatment is shown in fig. IV 7 and IV 8. In fig. IV 7 the cells have been prelabelled with ^{14}C -BUdR, incubated in the absence of label for 2 hours, treated with 10^{-3}M MMS and incubated in medium containing ^3H -thymidine for periods up to 19 hours. The DNA was isolated on alkaline Cs_2SO_4 and the heavy peak rebanded twice. In both the treated and control samples there are a large number of counts in the light strand, and these are proportional to the incubation time. However, 2 rebandings eliminates most of them. In the control this leaves very few counts at all, but in the MMS-treated cells there is a considerable amount of "repair replication" in the BUdR-labelled strand and this increases with incubation time. Up to 9 hours post-mutagen incubation time the counts are exactly coincident with the heavy peak as defined by the ^{14}C counts. However by 19 hours there is a slight shift towards a lighter density, the peak of the ^3H counts being one fraction higher than that of ^{14}C . This indicates that these newly-synthesised regions are increasing in length and that

(a) MMS 0-2 hours

(b) MMS 0-9 hours



(c) MMS 0-19 hours



(d) Control 0-19 hours

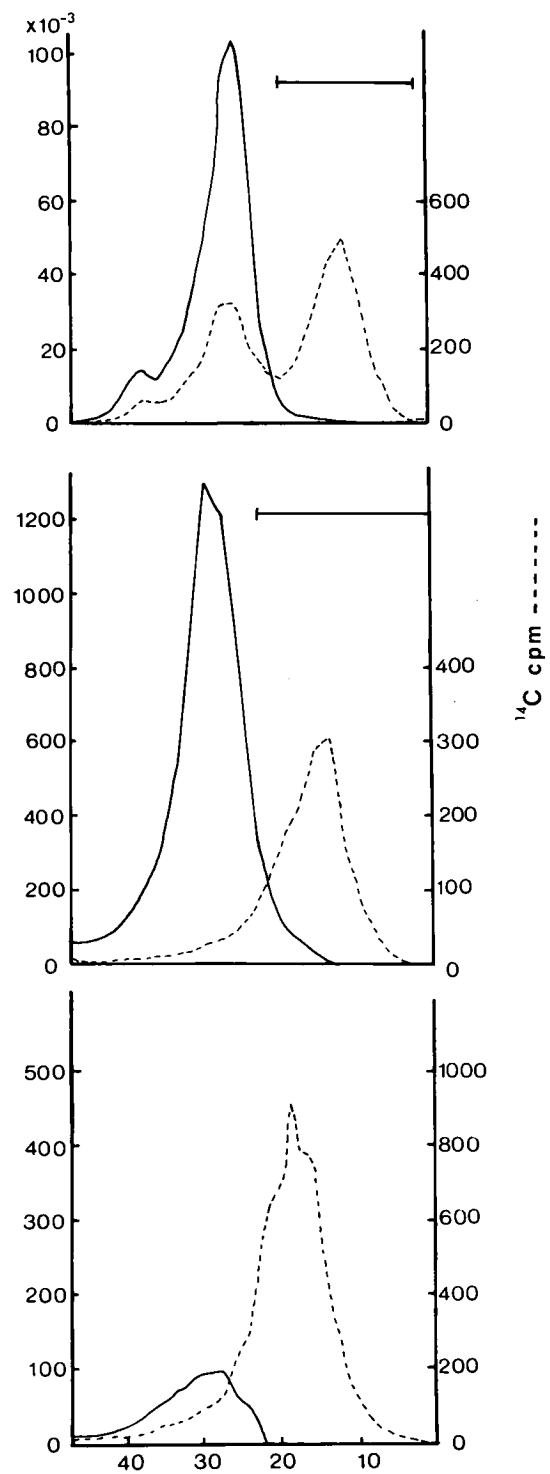
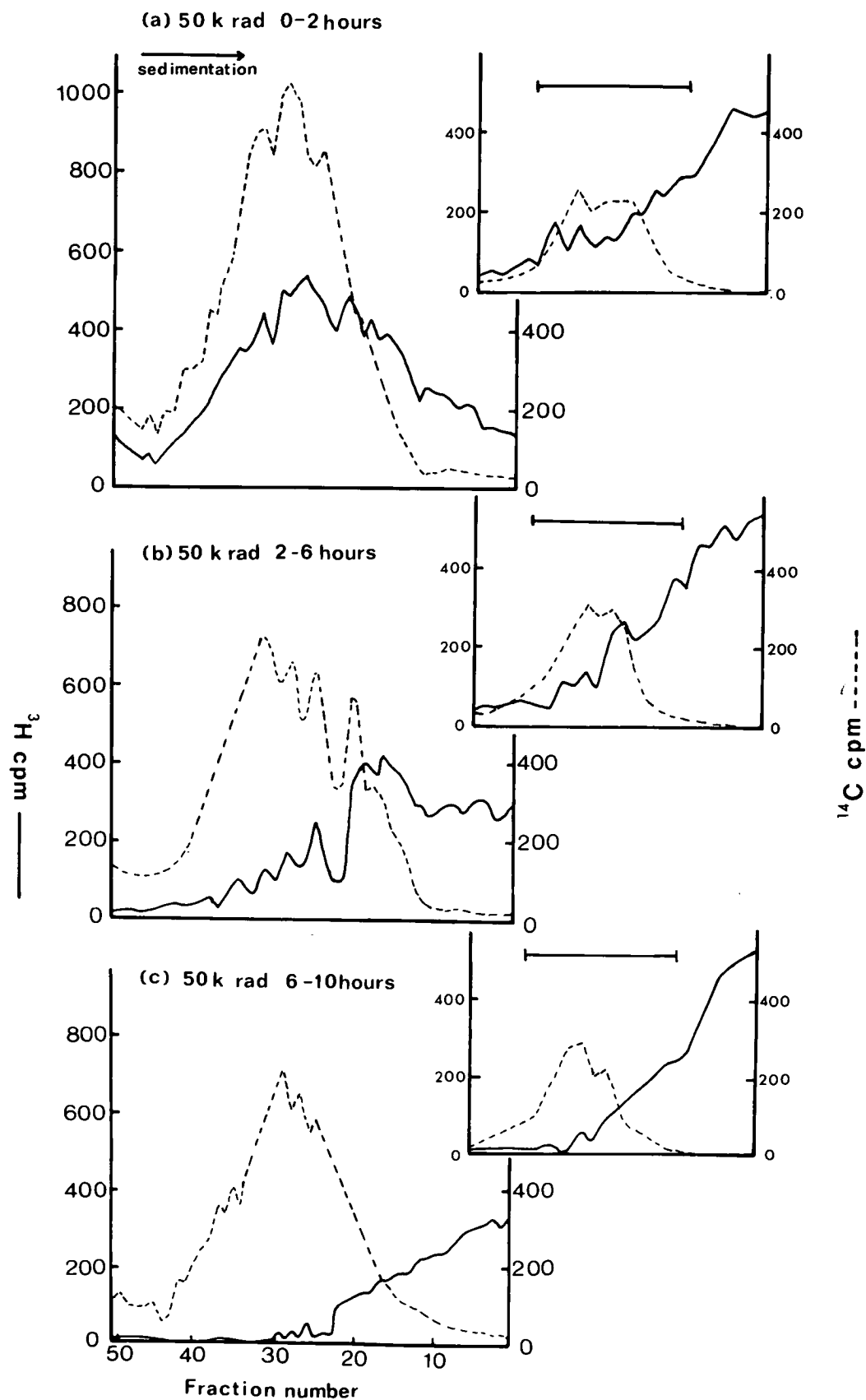


Fig. IV 7 Alkaline Cs_2SO_4 gradient profiles of DNA to show ^3H -thymidine uptake following incubation of cells in 10^{-3}M MMS. The cells were labelled for one generation in ^{14}C -CdR and for a second generation in ^{14}C -BUdR. Following mutagen treatment they were incubated in medium containing ^3H -thymidine for 2 hours (a) 9 hours (b) or 19 hours (c). An untreated control (d) was also incubated in a similar medium for 19 hours. The DNA was isolated on alkaline Cs_2SO_4 and the heavy peak (————) rebanded twice. Note the increased uptake of label with increasing incubation time and the slight reduction in buoyant density of the ^3H -labelled DNA at 19 hours post-treatment, its peak being one fraction lighter than that of the ^{14}C label (arrowed).



(d) 50 k rad 0-10 hours

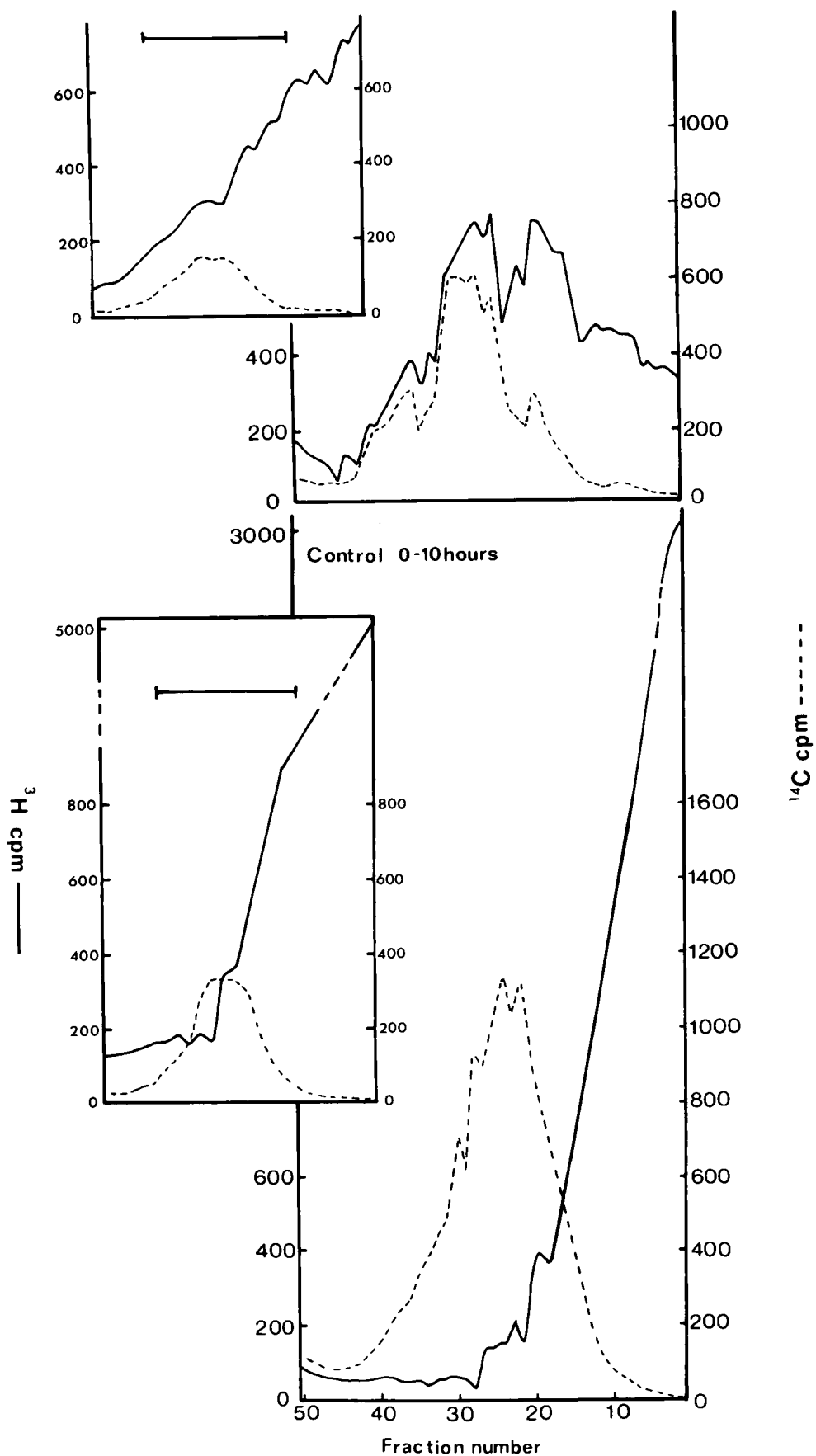


Fig. IV 8 BUdR uptake by stimulated cells prelabelled with ^{14}C -thymidine and irradiated with 50 k rads of γ -irradiation. The cells were incubated for various times in BUdR and ^3H -CdR and the DNA isolated on alkaline CsCl gradients (inset). The normal density region (————) was rebanded in a similar gradient.

- a) Incubated 0-2 hours in BUdR and ^3H -CdR post-irradiation
- b) Incubated 0-2 hours in BUdR and 2-6 hours in BUdR and ^3H -CdR
- c) Incubated 0-6 hours in BUdR and 6-10 hours in BUdR and ^3H -CdR
- d) Incubated 0-10 hours in BUdR and ^3H -CdR
- e) Untreated control incubated for 10 hours in BUdR and ^3H -CdR

Note the reduced rate of incorporation of ^3H into normal-density DNA with increasing incubation time and the bimodal distribution after 10 hour continuous incubation in ^3H -thymidine.

they are contributing to the buoyant density of the strand of which they are a part.

The amount of "repair replication" following γ -irradiation is shown in fig. IV 8. In this case the cells were prelabelled with ^{14}C -thymidine, incubated in BUdR for 2 hours and irradiated with 50 k rads. They were then divided into 4 (plus an unirradiated control) and incubated in the presence of BUdR plus ^3H -CdR either as a "continuous" or "pulse" label so that incorporation 0-2, 2-6, 6-10 and 0-10 hours post-irradiation could be estimated. The DNA was isolated on alkaline CsCl gradients and the normal region rebanded in a similar gradient (fig. IV 8). From the initial gradients it is obvious that $\underline{\text{S}}$ synthesis, evident at the bottom of the gradient, is very severely depressed by irradiation, being approximately 6 times greater in the control sample after 10 hours incubation. Rebanded shows that in the latter there are very few counts at the normal density compared with the irradiated cells, and that this level of counts in the irradiated sample increases very little beyond the 2-hour incubation period. There is however some synthesis 2-6 hours post-irradiation, mostly at a density intermediate between the normal and fully-substituted regions. This results in 2 equal peaks of ^3H counts at 10 hours post-irradiation.

3. MMS and γ -rays on Unstimulated Cells

Fig. IV 9 and IV 10 show similar experiments to that in fig. IV 8, but using unstimulated cells. Following 10^{-3}M MMS treatment (fig. IV 9) or 5000 rads of γ -irradiation (fig. IV 10) the cells were incubated in medium containing BUdR and "pulse" labelled with ^3H -CdR for the incubation periods shown. The DNA was then isolated on

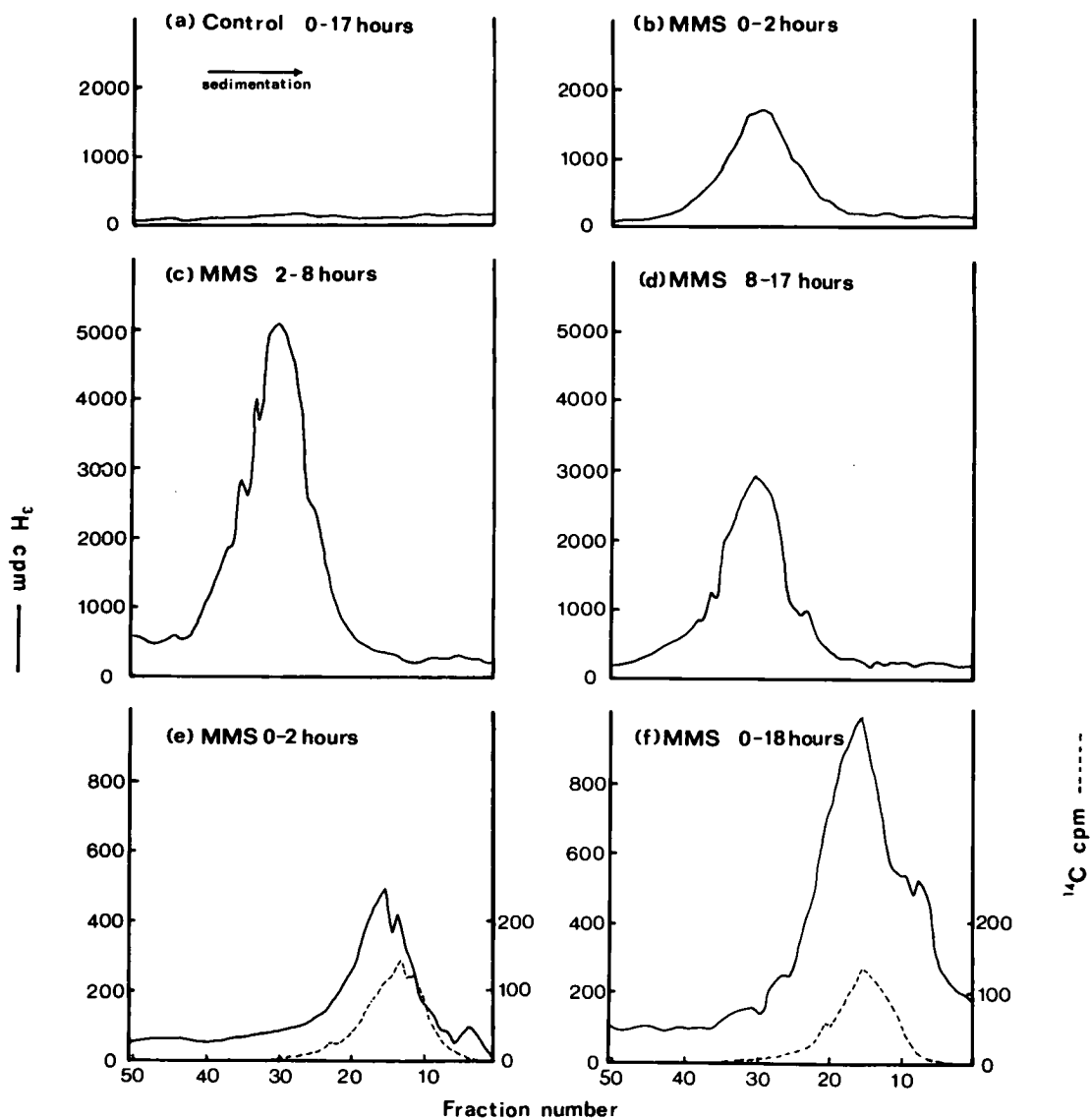


Fig. IV 9 Alkaline CsCl gradient profiles of DNA from non-stimulated cells incubated for 1 hour in 10^{-3} M MMS followed by BUdR and ^3H -CdR. Two experiments are shown. In the second (e and f) a ^{14}C marker DNA was added to the gradients which were of lower density than in the other experiments (57% CsCl).

- a) Untreated control incubated for 18 hours in BUdR and ^3H -CdR
- b) MMS-treated cells incubated 0-2 hours in BUdR and ^3H -CdR
- c) MMS-treated cells incubated 0-2 hours in BUdR and 2-8 hours in BUdR and ^3H -CdR
- d) MMS-treated cells incubated 0-8 hours in BUdR and 8-17 hours in BUdR and ^3H -CdR
- e) MMS-treated cells incubated 0-2 hours in BUdR and ^3H -CdR
- f) As e but cells incubated for 18 hours.

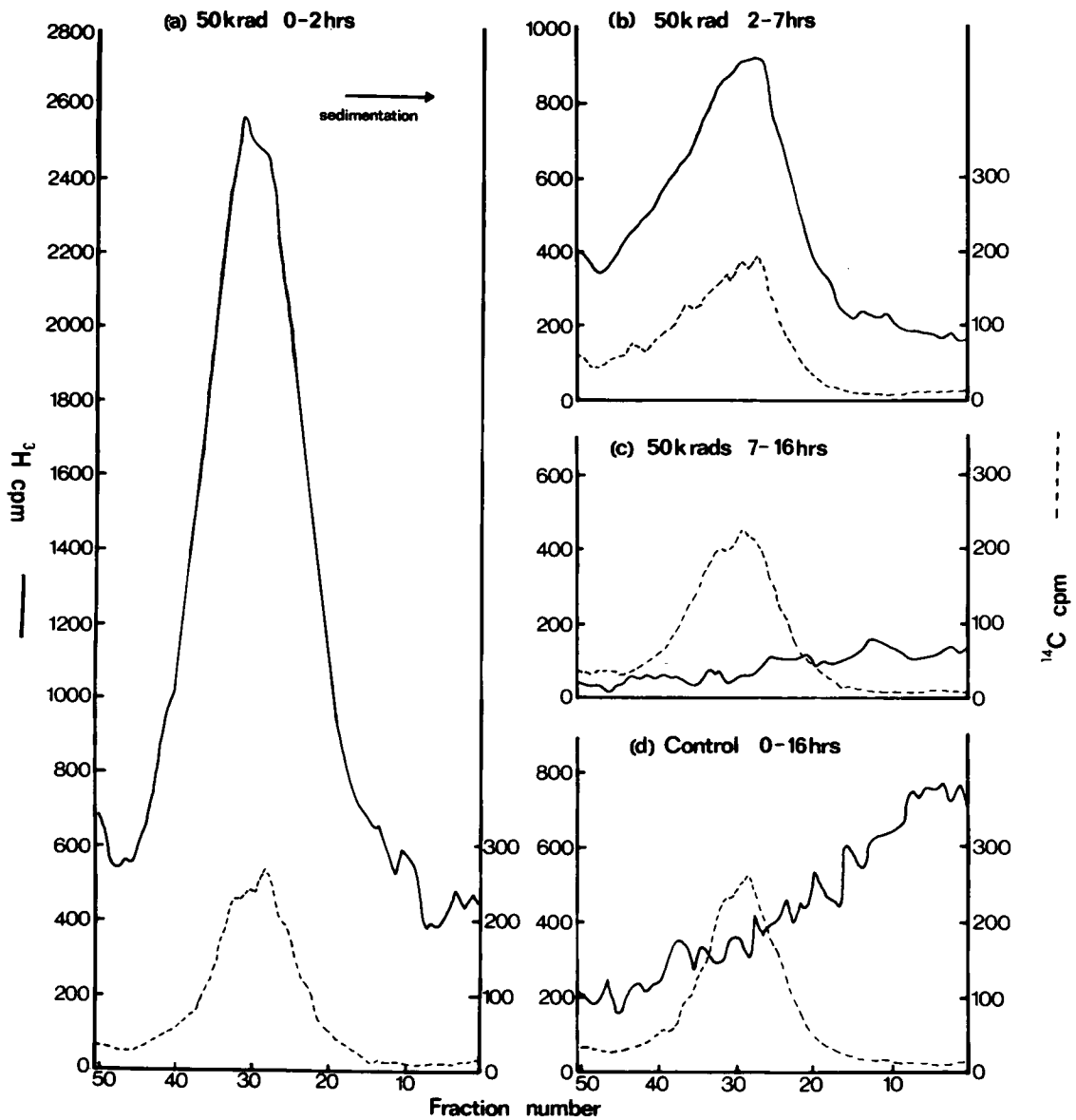


Fig. IV 10 Alkaline CsCl gradient profiles of DNA from non-stimulated cells following 50 k rads of γ -irradiation and incubated in BUdR and ^3H -CdR. A ^{14}C -marker DNA was added to the gradients to denote the position of normal-density DNA.

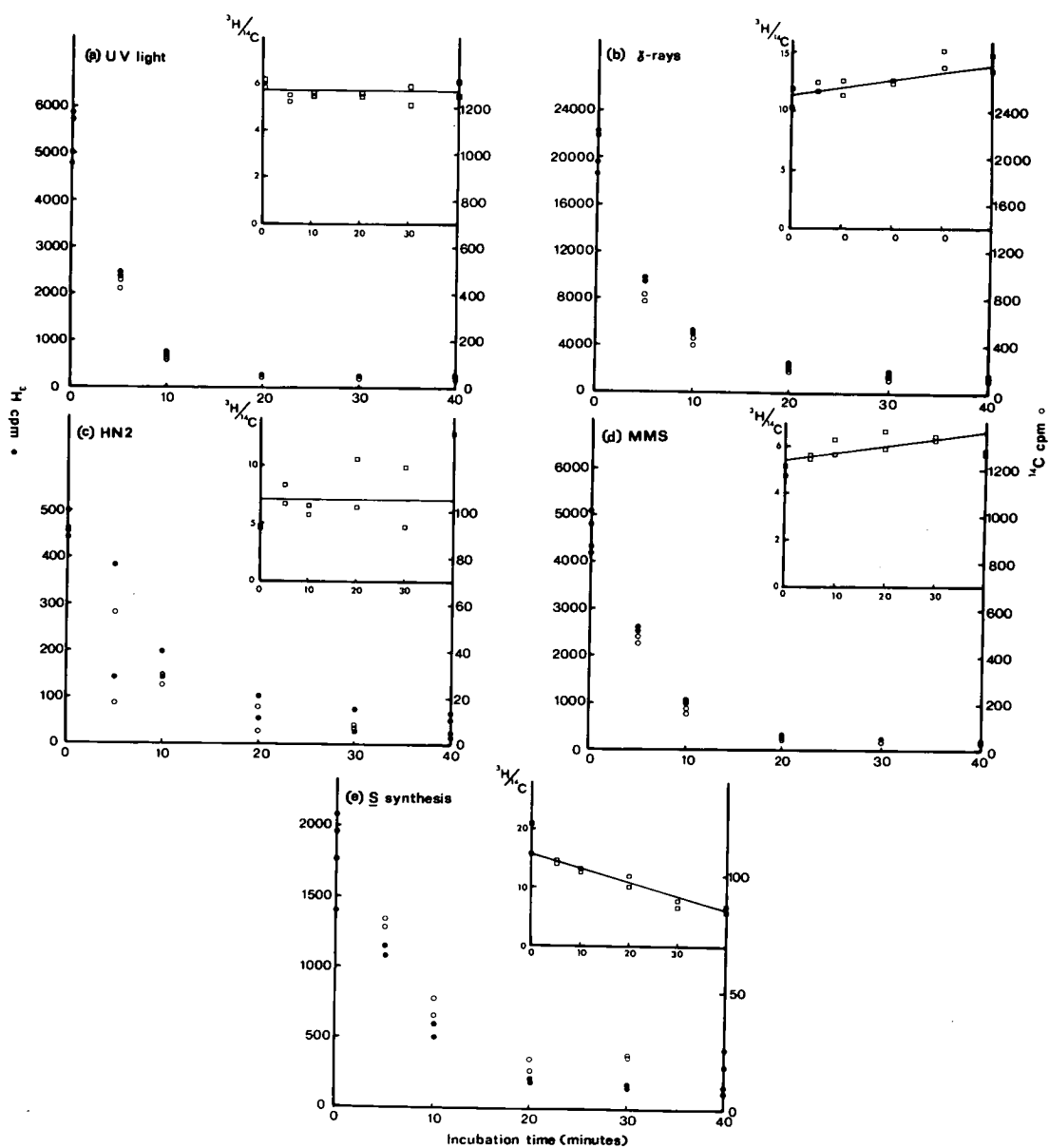
- a) Incubated 0-2 hours in BUdR and ^3H -CdR post-irradiation
- b) Incubated 0-2 hours in BUdR and 2-7 hours in BUdR and ^3H -CdR
- c) Incubated 0-7 hours in BUdR and 7-16 hours in BUdR and ^3H -CdR
- d) Unirradiated control incubated for 16 hours in BUdR and ^3H -CdR

alkaline CsCl gradients together with a ^{14}C -labelled marker DNA. These results show a very similar temporal pattern of synthesis to that for unstimulated cells for each of the mutagens. MMS-induced synthesis continues for at least 8 hours at a declining rate. There is no evidence for a shift in density of the main peak of ^3H (although this would not be evident in the absence of a marker), however, in the 0-18 hour sample there is a considerable shoulder on the heavy side of the peak indicating that some of the newly-synthesised regions may be of a considerable size.

The γ -ray data similarly show synthesis extending for the same length of time as in the stimulated cells, being complete within 7 hours post-irradiation. Unlike the data for stimulated cells, there is no evidence of a peak of ^3H at a density greater than normal, or even a heavy shoulder as in the MMS data. It is therefore likely that all of this DNA synthesis is in the form of small patches i.e. "repair replication". Unlike the MMS results the rate of synthesis declines very rapidly with time post-irradiation, being three quarters completed within 2 hours. A similar experiments to compare the uptake following 5 k rads with that due to 50 k rads shows the same temporal pattern at each dose, 60% of the synthesis being completed in the first hour post-irradiation.

4. Exonuclease Assays

In order to estimate the rate of degradation by venom phosphodiesterase of newly-synthesised regions of DNA relative to the overall rate of degradation the cells were prelabelled with ^{14}C -thymidine, treated with one of the mutagens, and incubated for 15-16 hours in medium containing ^3H -thymidine (see Chapter II section 10). The DNA was isolated on CsCl gradients, dialysed and denatured as described



Incubation time (minutes)

Fig. IV 11 The amount of residual DNA following incubation in venom phosphodiesterase, demonstrated as counts in TCA-insoluble material. The ^{14}C prelabel gives an estimate of the overall rate of degradation; the ^3H has been incorporated as a result of mutagen treatment (a) UV light, (b) γ -rays, (c) HN2, (d) MMS. (e) shows the pattern of uptake as a result of a 5' pulse of ^3H -thymidine during S phase synthesis (in the absence of mutagen). The insets show the relative rate of loss of ^3H compared with ^{14}C ; note that there is no difference in these rates for the UV and HN2 data, but that the ^3H is lost more rapidly when incorporated during S synthesis and more slowly following γ -rays and MMS treatment.

previously. In one case ^{14}C -labelled cells were given a 5-minute pulse label in ^3H -thymidine (in the absence of mutagen) in order to obtain DNA with ^3H attached to the growing points.

Venom phosphodiesterase is known to degrade DNA at the 3' hydroxyl end releasing 5' nucleotides (Williams *et al* 1961) so that ^3H counts concentrated at the 3' end will be preferentially degraded and ^3H at the 5' end will be lost more slowly. Attempts to use the complementary enzyme spleen phosphodiesterase were unsuccessful; the reverse result would have been useful confirmatory data. The concentration of phosphodiesterase was adjusted so that the DNA degradation could be followed over a 40-minute incubation period (see Materials and Methods). After 0, 5, 10, 20, 30 and 40 minutes incubation samples were assayed for TCA-insoluble counts in order to estimate the amount of DNA remaining intact. This is shown in Fig. IV 11. For each of the mutagens (plus ^3H incorporated as a result of S synthesis- Fig. IV 11e) the ratios of $^3\text{H}:^{14}\text{C}$ is plotted as an inset to the graphs. The relative rate of loss of each of the isotopes from the DNA has also been analysed by assuming an exponential model for the number of counts over the 40 minute incubation period. Under this model, the regression co-efficient in a log (counts) on time regression gives a measure of the rate of change of counts with time. The differences in the rate co-efficients between the loss of ^3H and the loss of ^{14}C for each mutagen is shown in Table IV 1. In the case of UV and HN2 this does not differ significantly from zero. However in the other 3 cases (S synthesis and γ -ray- and MMS-induced synthesis) the magnitude of the difference is significant ($P = 0.05$), being greatest when the ^3H is incorporated during S synthesis and suggesting that some of the synthesis induced by γ -rays and MMS could be accounted

Table IV 1 The differences in the rate coefficients for the loss of ^3H , incorporated as a result of mutagen action, as compared with the overall rate of degradation of the DNA (measured as the loss of ^{14}C) by venom phosphodiesterase.

Experiment	Magnitude of rate Difference coefficient	Standard Deviation
UV	0.0039	0.002
HN2	0.011	0.006
γ -rays	0.007	0.002
NMS	0.0084	0.002
5' pulse	0.03	0.002

Table IV 2 The amount of each isotope remaining in the DNA following incubation in venom phosphodiesterase for 40 minutes. The estimates are derived from log (counts) on time regressions of the data in Fig. IV 11.

Treatment	Isotope	% residual after 40 minutes
NMS	^{14}C	2.9
	^3H	5.1
UV light	^{14}C	3.5
	^3H	3.5
γ -rays	^{14}C	5.1
	^3H	6.8
HN2	^{14}C	12.0
	^3H	12.0
<u>S</u> synthesis	^{14}C	20.0
	^3H	7.4

for by end addition of nucleotides. However a large part of the ^3H incorporated as a result of γ -ray and MMS treatment of the cells is evenly distributed along the DNA molecule in a manner resembling that following UV irradiation (and HN2) and it is therefore likely to be due to "repair replication".

In Table IV 2 the amount of each isotope remaining in the DNA after 40 minutes incubation with the enzyme, estimated from the regressions, is shown for each case. It is evident that there is preferential labelling of the 3' end of the DNA during S synthesis, only 7% of the ^3H remaining at this time compared with 20% of the total (as estimated by ^{14}C). In contrast, for the γ -ray and MMS data, the ^3H is concentrated at the 5' end, being lost at a slightly slower rate and is consequently not being incorporated as a result of residual S synthesis. This difference between incorporation due to S synthesis and that due to γ -rays and MMS is also evident from the difference in slope for the graphs of $^3\text{H}:^{14}\text{C}$ (see insets of Fig. IV 11).

DISCUSSION

The experiments in this chapter were aimed at characterising the mutagen-induced unscheduled DNA synthesis demonstrated in the previous chapter by ^3H -thymidine incorporation into TCA-insoluble cell extracts. It was shown that the patterns of synthesis differed in the case of UV and HN2 as compared with MMS and γ -rays and it was felt likely that similar differences might be apparent in the amount of "repair replication" following these mutagens.

1. UV irradiation

The results for "repair replication" following 700 ergs/mm^2 UV irradiation, as demonstrated by ^3H -thymidine incorporation into

BUdR-labelled DNA, or BUdR incorporation into normal-density DNA, show that all the synthesis is completed within 6 hours post-irradiation and that the peak of ^3H counts coincides at all times with the ^{14}C counts defining the position of the DNA strand. This shows that the newly-synthesised regions are short compared with the pieces of DNA on the gradient and that this synthesis conforms to the definition of "repair replication". This temporal pattern of "repair replication" is similar in both stimulated and non-stimulated (G_1) cells and very closely resembles that for unscheduled DNA synthesis in unstimulated cells demonstrated in the previous chapter; at 350 ergs/mm^2 being 60% completed after 2 hours and almost over within 6 hours. Cleaver (1970a) showed for repair replication at 35 ergs/mm^2 a very similar temporal pattern to the unscheduled DNA synthesis data at low UV doses, the rate declining sharply over the first 4 hours post-irradiation.

2. γ -rays and MMS treatment

For the γ -ray and MMS data it is not so easy to equate "repair replication" with unscheduled DNA synthesis demonstrated in Chapter III. Following MMS treatment of unstimulated cells, the incorporation of label into the DNA continues for at least 8 hours in a manner resembling the previous data. Almost all the counts exactly coincide with the main DNA peak, although there is some evidence of a heavy shoulder indicative of relatively large regions of newly-synthesised material. The two experiments shown in Fig. IV 9 give different relative amounts of synthesis at the various time intervals post-treatment but there is evidence of a faster rate of synthesis initially.

Following γ -irradiation of the same unstimulated cells, a different picture emerges, all the "repair replication" is completed

within 7 hours and 60% of this is over within the first hour. This holds for synthesis induced by both 5 K rads and 50 K rads and is virtually identical to the results of Brent and Wheatley (1971) for G_1 -phase HeLa cells X-irradiated with 4400 rads.

The results for "repair replication" induced by these agents in stimulated cells are similar to these for non-stimulated. The MMS-induced synthesis again continues (in BUdR-labelled DNA) for at least 9 hours at a declining rate and after this time there is evidence for a slight shift in density of the newly-synthesised regions relative to the main DNA band. Fox and Ayad (1971), working on P388 lymphoma cells, have studied the temporal pattern of "repair replication" after MMS in the presence and absence of hydroxyurea. In this system the induced synthesis, measured as ^3H -BUdR uptake into normal-density DNA, is completed within 8 to 10 hours and in the absence of hydroxyurea all the "repaired" DNA has replicated within 15 hours even at levels of MMS which reduce survival to 5%. However when hydroxyurea is present and there is therefore little semiconservative replication, they claim that the main DNA band, containing ^3H -BUdR, has an increased buoyant density, suggesting a very large incorporation of BUdR into all parts of the genome.

The duration of synthesis following γ -irradiation in stimulated cells is also similar to that in G_1 cells, being completed by 6 hours post-irradiation. However in this case there are 2 populations of newly-synthesised molecules. In one, the ^3H counts are coincident with the ^{14}C counts and therefore represent true "repair replication". Most of this synthesis is completed within 2 hours post-irradiation. Throughout this 6-hour period synthesis of longer molecules is shown

by a peak of ^3H at a slightly greater density. Continuous incubation of the cells in ^3H -CdR and BUdR for 10 hours shows that these 2 types of synthesis are present in equal amounts and that the "repair replication" of the first 2 hours is not affected by the subsequent synthesis, i.e. these regions do not continue to increase in length with time. Painter and Young (1972) have very recently similarly shown that during a 3-hour post-irradiation incubation P388F lymphoma cells synthesised 2 populations of DNA molecule; one due to "repair replication" and a second representing much longer regions of substitution. The latter showed a much greater density shift than in the present work. However this may be due to greater shearing in the preparation of their DNA which included incubation with pronase and ribonuclease and an additional rebanding in CsCl gradients.

Fox et al (1970) have described a form of hydroxyurea-resistant synthesis following X-irradiation of P388 lymphoma cells. Autoradiographic studies by them suggest that it is restricted to cells in S phase and it would be interesting to see if the synthesis of large patches, restricted to stimulated cells, in this work showed a similar distribution. In this connection it may also be significant that PHA-stimulation of lymphocytes results in an increased amount of unscheduled DNA synthesis in response to ionizing radiation (Spiegler and Norman 1969) and UV light (Darzynkiewicz 1971a; MacKay and Evans 1972). A reversible reduction in DNA "repair" levels associated with differentiation and loss of cellular proliferation has also been demonstrated in hen erythrocytes (Darzynkiewicz 1971b) and skeletal muscle cells (Stockdale 1971).

This difference in the pattern of "repair replication" following

γ -irradiation and MMS treatment is somewhat surprising in view of the similarity in the temporal patterns of unscheduled DNA synthesis induced by these 2 agents. In fact there is no evidence of any induced DNA synthesis for more than 8 hours after γ -irradiation and this contrasts markedly with the results obtained in similar experiments where label was incorporated into TCA-insoluble material for up to 22 hours post-irradiation (see Chapter III). It is possible that this latter result is due to the incorporation of label into other macromolecules such as into RNA, although its dose-dependence makes this explanation unlikely. The other possibility is that not all of the DNA is being recovered on the gradients. Newly-synthesised DNA could be closely bound to polymerases forming a complex with proteins that are precipitated. This latter explanation could only be confirmed by carefully quantifying the ^3H counts at each stage in the isolation procedure.

However, it is interesting that in the case of both the γ -ray-induced unscheduled DNA synthesis and "repair replication", there is a much faster rate of synthesis during the first hour post-irradiation and it has already been suggested for the unscheduled DNA synthesis data that this might be associated with the rejoining of single-strand breaks with the insertion of one or two nucleotides per lesion. These results for "repair replication" are consistent with this. The continued DNA synthesis lasting for 6 to 7 hours post-irradiation and evident (in the stimulated cells) as ^3H counts at a slightly greater density would, in this case, represent an entirely different type of reaction. The less marked initial fast rate of "repair replication" following MMS treatment is consistent with the unscheduled DNA synthesis

data where it was suggested that this was a consequence of the types of damage induced; strand breakage being a primary lesion following X-irradiation, but a secondary reaction following methylation resulting from depurination of the affected bases (Strauss and Hill 1970). In the case of the latter, rejoining could be a more extended process and synthesis associated with it would be expected to have a correspondingly longer temporal pattern. The second type of reaction, continuing for longer than that following γ -irradiation, also results in the synthesis of relatively long molecules. However these appear to be more closely associated with the "repaired" regions as they do not band as a second ^3H peak.

3. The significance of the synthesis to known types of damage

That the type of induced synthesis may depend on the damage produced by the mutagen is reflected in the differing amounts of repair in normal and BU-substituted DNA. BU has been known for some time to sensitise cells to UV irradiation (Djordjevic and Szybalski 1960) and it is known that UV irradiation of DNA in which thymine is replaced by BU results in DNA strand breakage (Hutchinson and Koehnlein 1967). Cleaver (1968a) showed that following 1000 ergs/mm^2 70% of the fresh bases were incorporated into the BU strand of HeLa cells with hybrid DNA and this agrees well with these results for 700 ergs/mm^2 where S synthesis is negligible (see upper Fig. IV 6). It differs markedly, however from the results for MMS. Here the majority of the incorporation is into the normal DNA strand and suggests that the BU is having a protective effect, possibly due to its greater size compared with thymine, preventing alkylation of other bases. Following γ -irradiation where damage is predominately strand breakage, and where this

discriminative effect would presumably not apply, the discrepancy between the 2 strands is much smaller and the number of counts in the light strand is of the same order as in the control cells. It is probably significant in this connection that the temporal pattern of UV-induced synthesis in the BU-substituted strand resembles that of normal-density DNA and may thus invalidate the argument that the difference in temporal patterns between UV- and HN2-induced synthesis and the synthesis due to γ -rays and MMS is simply a consequence of strand breakage in the latter but not the former.

These same experiments also demonstrate that the amount of repair in the BU strand after each of the mutagens is the same when this is the newly-synthesised strand or in the template strand. This is in contrast with the results for DNA strand rejoining of P388F lymphoma cells following MMS treatment giving 50% survival (Fox and Fox 1969). In this case the newly-synthesised DNA increases in molecular weight during a 2-hour post-treatment incubation, possibly as a result of strand rejoining or due to synthesis onto ends of fragmented DNA, but that template-labelled DNA does not have this ability. If this difference also applied to the DNA in the lymphocytes used in this study, it would suggest that "repair replication" was of little significance in the process of strand rejoining, if in fact this is what is demonstrated by Fox and Fox.

The data presented here, at least for the γ -ray induced synthesis, is not inconsistent with the hypothesis that during the first hour post-treatment the mutagen-induced synthesis is "repair replication", and could be associated with the rejoining of single-strand breaks. Donlon and Norman (1971) have already shown a similarity in the

kinetics of unscheduled DNA synthesis and strand rejoining. The experiments with exonuclease were aimed at throwing light on the significance of the more prolonged synthesis. The assumption that ^3H -thymidine incorporated into DNA by "repair replication" would be lost at the same rate as the ^{14}C used as a prelabel, is confirmed by the UV data. This also holds for HN2-induced synthesis. By comparison, ^3H -thymidine incorporated during a 5 minute pulse in S synthesis, presumably onto the growing points, shows a different pattern, the ^3H being lost preferentially from the 3'-hydroxyl end of the DNA. The MMS and γ -ray data are different again, the ^3H being preferentially untouched i.e. it is concentrated at the 5' end. However this difference in the rates of degradation of ^3H and ^{14}C is small (but significant) compared with the results for S synthesis and suggests that only part of the reaction is comparable with this. It is unlikely that this incorporation is due to residual S synthesis as this is always in the 5' to 3' direction (Kornberg 1969). It is therefore possible that the extended synthesis following MMS and γ -rays could, at least in part, be explained as a form of end addition possibly within the gaps, and it would be interesting to compare the effect of exonuclease degradation on each of the 2 peaks of γ -ray-induced synthesis.

On the strength of the unscheduled DNA synthesis data where synthesis appears to continue beyond the 15-hour incubation period used in the preparation of the DNA, it could be argued that the apparent end-addition is in fact due to regions in the process of being repaired and awaiting ligase action. This would mean that polymerisation had occurred in the 3' to 5' direction, a fact inconsistent with the data

of Kelly et al (1969). They showed that DNA polymerase from E.coli is able to excise UV-induced pyrimidine dimers and to insert new nucleotides into the DNA in the 5' to 3' direction. It is of course possible that a different type of enzyme system is concerned with damage following γ -rays and MMS and the results for the initial rates of synthesis following each of the mutagens in Chapter III indicate that different enzymes are involved in the rate-limiting step in each case. The data of Painter (1970) also suggest that this result cannot be explained in terms of incomplete repair at some of the damaged sites. He could detect no difference in the rates of degradation of uniformly-labelled HeLa cell DNA as compared with cells given 10^5 rads and a 3-hour label (at which time "repair replication" is incomplete, see Fig. IV 10). It is likely, though, that differences of the order described in the present work would not be apparent unless the DNA were prelabelled in order to give a more exact estimate of the overall rate of degradation.

The experiments described in this chapter were designed to characterise the mutagen-induced synthesis described in Chapter III and in particular to explain the differences in the temporal patterns between that induced by UV and HN2 compared with that by γ -rays and MMS. It has been clearly demonstrated that all of the synthesis induced by UV light is due to "repair replication". There is also a similarity in the patterns of unscheduled DNA synthesis and "repair replication" following MMS treatment; both show an initial fast reaction, possibly associated with strand rejoining, and, in addition, a more prolonged, somewhat slower rate of synthesis that could represent "repair" of alkylated bases. The evidence from the density gradient

profiles suggests that these regions could be fairly extensive. Following γ -irradiation a similar initial fast reaction is evident and this also appears to be due to "repair replication". The subsequent extensive synthesis demonstrated in Chapter III is not apparent as "repair replication" although there is some synthesis in the form of longer stretches of DNA. The exonuclease experiments do not exclude the possibility that the γ -ray-induced synthesis at longer incubation times post-irradiation is "repair replication" and it is suggested that not all of the newly-synthesised DNA is being recovered on the gradients.

V. THE IDENTIFICATION OF "REPAIR" - DEFICIENT

HUMAN GENOTYPES

INTRODUCTION

In the introduction it was shown that the radiosensitivity of a cell was at least partly genetically determined. Here I intend to consider certain implications of this and also consider how environmental factors can modify or mimic the situation, thereby giving some indication of the mechanisms involved.

Sensitisation of Micrococcus radiodurans to X-irradiation by 5-bromouracil (BU) illustrates this. Progressive replacement of the thymine in cellular DNA by BU results in progressive removal of the shoulder from the anoxic survival curve. This is paralleled by interference with DNA degradation that normally follows X-irradiation and when the level of BU incorporation is sufficient to eliminate the shoulder, the degradation-excision system is inoperative, (Lett et al 1970). Similarly, genetic defects affecting this system, such as uvr⁻ mutant described earlier, reduce survival by preventing excision repair (Boyce and Howard-Flanders 1964; Setlow and Carrier 1964).

Survival and Mutation Frequency after Irradiation

Increased viability and decreased mutation frequency of E.coli or yeast occurs if suspensions are kept in non-nutrient media for some hours after irradiation and before plating (Patrick et al 1964; Castellani et al 1964). This phenomenon is known as "liquid holding" and is thought to slow growth, allowing time for recovery to take place. In Paramecium changing the medium initiates DNA synthesis and this technique has been used to show that at a given dose level the greater the time between irradiation and S, the fewer the mutations

produced (Kimball 1961). Treatments known to modify the mutation frequency are only effective prior to the first post-irradiation chromosome duplication (Kimball et al 1957).

The UV-sensitive mutants of E.coli have also clarified the position with regard to stable mutation induction, showing that the damage responsible is potentially capable of being repaired. If phage is irradiated prior to the infection of the host cell, its survival is dependent on the genotype of the host. In wild-type cells a certain amount of recovery of the phage is evident and this phenomenon is known as host cell reactivation. E.coli WP2 is unable to repair the damage in irradiated phage (hcr^-) and at low doses, itself shows a low level of survival with a large amount of mutation. At these doses no mutations are induced in the WP2 parental strain (Hill 1965). As in E.coli B_{S-1}, the survival of irradiated bacteriophage is very low and it is probable that neither strain can excise dimers. However WP2 shows a certain degree of tolerance and it is postulated that replication proceeds past the dimers with a high degree of inaccuracy (Witkin 1966).

Mutation frequency and killing after low doses of UV can also be increased by post-treatment with caffeine (Witkin 1958) or acriflavine (Witkin 1961). Both compounds in themselves are non-mutagenic under the conditions used, but they markedly decrease the rate of excision of thymine dimers from the DNA of UV-irradiated wild-type bacteria (Setlow 1964; Shimada and Takagi 1967). The effect of these agents on radiation-induced killing is less pronounced in hcr^- strains, consistent with the reduced involvement of the excision repair system. However, mutation frequency is slightly reduced and it is postulated

that a second repair mechanism is responsible for eliminating at least some of the pre-mutational lesions (Clarke 1967; Sideropoulos and Shankel 1968; Horneck-Witt and Kaplan 1968). The slightly enhanced killing still evident in irradiated *hcr*⁻ mutants exposed to caffeine is of the same order as the reduction in recombination in the presence of caffeine, and recombination repair (see Introduction) is thought to be affected; its efficiency being reduced, but its accuracy increased (Witkin and Farquarson 1969). This is supported by the reduced mutation frequency in *rec C*⁻ mutant of *E.coli* following UV irradiation (Witkin 1969) and the reduction by caffeine of recombination and UV-induced mutations in *Schizosaccharomyces* (Loprieno and Schupbach 1970).

In mammalian cells, where caffeine reduces the mutation frequency and survival after UV irradiation (Trosko and Chu 1971), recombination repair is also thought to be involved as there is no effect on dimer excision (Regan et al 1968) or repair replication (Cleaver 1969b). However, the newly-synthesised (semi-conservative) DNA following UV irradiation of Chinese hamster cells has interruptions at approximately the same frequency as that of unexcised thymine dimers in the parental strand (Cleaver and Thomas 1969). The molecular weight of these strands increases with incubation time in a manner similar to that following recombination repair in bacteria (Rupp and Howard-Flanders 1968; Rupp et al 1971) and Cleaver and Thomas have shown that this mechanism (in mammalian cells) is inhibited by caffeine.

Caffeine also increases the frequency of chromosome aberrations following the γ -irradiation of barley (Yamamoto and Yamaguchi 1969) and the exposure of *Vicia faba* root cells to a chemical mutagen

(diepoxybutane- Swietlinska 1971).

Radiosensitivity and Recombination

An association between a reduced ability to undergo genetic recombination and an increased UV-radiosensitivity was first demonstrated by Clark and Margulies (1965) for rec^- strains of E.coli K12. Subsequently the extreme sensitivity of the double mutant $uvrA^- recA^-$ led to the idea of a second dark repair system in which an intact DNA strand is formed from undamaged regions of parental and daughter strands in a manner comparable to genetic recombination (see Introduction). This system has not been demonstrated in eukaryotic cells although there is considerable circumstantial evidence to suggest it exists (see above), including the association between radiosensitivity and reduced crossing-over at meiosis. Reduced sporulation has been described in a radiosensitive strain of yeast (Cox and Parry 1968) and a desynaptic mutant of *Drosophila* (c3G) showing reduced recombination has been shown by Watson (1969) to be abnormally sensitive to X-irradiation. Barley plants homozygous for a desynaptic gene and showing a reduction in chiasma frequency at meiosis, were found by Riley and Miller (1966) to have an increased sensitivity to the induction of chromosome exchanges following X-irradiation. However these observations on barley were not confirmed in more extensive experiments carried out by Swietlinska and Evans (1970).

Genetic Factors and Predisposition to Malignancy

One of the most widely documented groups of malignant diseases in man are the leukaemias and the association of certain kinds of leukaemia with genetic factors is well established. Several groups of people are known to be particularly prone to the development of leukaemia, including the identical twin of an existing case (Miller

1968); sufferers of the genetically transmitted diseases Fanconi's anaemia, Bloom's syndrome and ataxia telangiectasia (Garriga and Crosby 1959; Peterson et al 1964; Sawitsky et al 1966; Miller 1967) and individuals with Down's syndrome (Krivit and Good 1957; Miller 1967). An association between chronic granulocytic leukaemia and an abnormal chromosome in the G group (Philadelphia chromosome) was first described by Nowell and Hungerford (1960). It is present in granulocytic cells of the bone marrow and was subsequently shown to be chromosome 22 (O'Riordan et al 1971) in which part of the long arms are deleted. Deletion of the short arms of this chromosome was first described by Gunz et al (1962) who named this particular chromosome the Christchurch chromosome. Its association with leukaemia has since been disputed (Court Brown 1964; Juberg and Jones 1970) and, unlike the Philadelphia chromosome, it is present in all tissues and segregates normally among the progeny of carriers.

In addition to these inherited conditions, acquired genetic damage can confer a predisposition to malignancy. Chromosome breakage (characteristic of Bloom's and Fanconi's syndromes) is also associated with exposure to ionizing radiation (see Introduction) and various chemical agents (Evans 1970) including benzene (Tough and Court Brown 1965). Hiroshima survivors and radiotherapy patients show a high incidence of leukaemia (Brill et al 1962; Court Brown and Doll 1965) and benzene is also thought to be leukaemogenic (Vigliani and Saita 1964).

That cells can normally tolerate and recover from a certain amount of environmentally induced damage in their DNA is illustrated in the case of Xeroderma pigmentosum. Individuals with this condition

develop neoplasms on exposed parts of the body (Cockayne 1933) and the demonstration by Cleaver (1968) that cells from such patients are unable to undergo unscheduled DNA synthesis after UV irradiation suggests that a certain amount of damage is normally repaired and that an inability to do this results in malignant transformation (see Chapter IV). It also seems likely that inhibition of DNA repair is responsible for the action of co-carcinogens (Gaudin et al 1971).

Criteria for the Selection of Potential Mutants

There is therefore a complex relationship between cell survival, chromosome damage, mutation induction, malignant transformation and the ability to excise damaged regions of DNA following environmental insult. They are each subject to modification, not least by the cells inherent capacity to repair genetic damage. If, as we believe, unscheduled DNA synthesis is a form of repair process, an association between it and these characters would not be surprising. In considering this association in relation to man, it seemed possible that human genotypes with a reduced capacity for unscheduled DNA synthesis might manifest at least one of the following criteria:

- a) An inherited disposition for an elevated incidence of spontaneous chromosome rearrangements evident in PHA-stimulated lymphocytes, bone marrow and fibroblasts.
- b) A heritable predisposition to malignant transformation as a consequence of an increased spontaneous mutation rate.
- c) Reduced recombination at meiosis as manifested by a reduction in chiasma frequency, possibly comparable with rec^- bacteria.
- d) Abnormal sensitivity to sunlight which could result in an inability to repair UV-induced lesions.

e) An inherited tendency for reduced growth (stunting) and/or premature ageing. This could also affect proliferating tissue such as bone marrow resulting in haematological disorders.

Conditions under Investigation

1. Bloom's syndrome A rare condition the clinical features of which were first described by Bloom (1954) and are typically stunted growth with low birth-weight following a full-term gestation and telangiectatic erythema evident during the first three years and aggravated by exposure to sunlight. Its occurrence in sibs and association with consanguinity of parents (Szalay 1963) indicates that a recessive autosomal gene is responsible. However, its prevalence in males (more than $\frac{3}{4}$ of the cases - Bloom 1966) may mean that the situation is more complicated. Other characteristic features are chromosome damage in lymphocytes (German et al 1965) and fibroblasts in long-term culture (German and Crippa 1966), and increased incidence of leukaemia (Sawitsky et al 1966).

The patient considered in this study was a six-year old male exhibiting all these features.

2. Fanconi's aplastic anaemia A rare condition, the clinical features of which and its familial occurrence were first noted by Fanconi (1927). Typically, the patients are short with skeletal deformities, abnormal skin pigmentation and mental retardation. The abnormalities of the peripheral blood may not appear until late in the first decade of life. Consanguinity of parents (Fanconi 1964) confirmed its autosomal recessive inheritance. As in Bloom's syndrome chromosome aberrations (Schroeder et al 1964; Bloom et al 1966) and predisposition to leukaemia are characteristic. Clinical relapses are often associated

with viral infections (Swift and Hirschhorn 1966) and it is interesting in this connection that fibroblast from these patients are ten times more easily transformed by SV40 virus (Todaro et al 1966). Todaro also found a slight increase in heterozygous individuals but this was not confirmed by Young (1971).

The individuals considered here are a family in which the two elder children have Fanconi's anaemia (child A and child B) and the two younger ones (child C and child D) have so far no evidence of the disease. Transformation studies with SV40 also suggest that the younger children are normal (or heterozygous). The parents are assumed to be heterozygous. In addition, two other confirmed cases have been considered.

3. Xeroderma pigmentosum Although apparently normal at birth these individuals soon develop erythema and freckling on exposure to sunlight (Cockayne 1933). The pigmentation increases with age and malignant tumors develop resulting in early death (usually before 29 years of age). I have already mentioned the demonstration by Cleaver (1968) of reduced repair replication following UV irradiation and Bootsma et al (1970) have correlated this with the severity of the disease, no synthesis occurring in the very severe form (de Sanctis-Cacchione syndrome) which is accompanied by neurological disorders (de Sanctis and Cacchione 1932).

Cockayne assumed this condition to be due to a single recessive gene although freckling in relatives of patients was independent of their hair colour and these were assumed to be heterozygous. Partial sex-linkage was suggested by Haldane (1936) and Macklin (1944). The patient from whom I have obtained blood samples (Douglas McPherson)

has already been described by Anderson and Begg (1950). His is a particularly mild case and he was aged 51 at the time of study. His current lesions are minor but a more severe expression was evident when the patient spent some time in North Africa. Heterozygous expression of the gene in this patient's relatives is not marked and appears to be dependent on the genetic background.

4. Christchurch Chromosome (Ch') The association between malignant diseases and genetic defects, and in particular the association between leukaemia and deletions in chromosome 22 have already been referred to. Although deletions in the long arms (Ch' chromosome) are not generally considered to be significant in this connection, it was felt that lymphocytes from such individuals were worth investigation. Even if an association between defective DNA repair and a predisposition to leukaemia could not be conclusively established, there was always the possibility of locating loci concerned with repair by looking for a deficiency in cells with missing genetic material. In the case of alkaline phosphatase, reduced levels are associated with a deletion in the long arms of 22 (Ph' chromosome) and the genetic information for its synthesis has been assumed by Valentine and Beck (1951) to be located there although this assumption has not been substantiated. Blood samples from two individuals with Ch' chromosomes were available. The first individual considered (H.W.) had a Ch' chromosome and mediastinal and retroperitoneal fibrosis which was subsequently found to be non-malignant. It was also possible to consider these two factors independently. Individual R.B. had a Ch' chromosome and bladder cancer; whereas a third individual, E.H., was chromosomally normal but had similar fibrosis. Each was compared with controls

matched for age and sex and where necessary radiotherapy treatment was taken into account.

5. Achiasmate males Mutations that affect the pairing of chromosomes at meiosis have been described for many organisms (see review by Riley and Law 1965) and in each case have been ascribed to the homozygosity of recessive alleles. These mutants are of two types: asynaptic, where the chromosomes fail to pair at first meiotic prophase; and desynaptic where chiasma formation is severely reduced despite the occurrences of synapsis. The evidence is generally consistent with the view that chiasma formation can be equated with the occurrence of recombination (Brown and Zohary 1955; Jain and Basak 1963). In both the Drosophila mutant (Watson 1969) and the barley strain (Riley and Miller 1966) described earlier, in which there is increased radiosensitivity and reduced recombination, desynapsis is evident, i.e. the repair mechanism is associated with a possible abnormality in the breakage and rejoining of the DNA backbone and not with the initial stages of chromosome pairing on which this is dependent. In looking, then, for human mutants with a reduced capacity for repair, individuals with a reduced chiasma count as a result of desynapsis have been selected, and unscheduled DNA synthesis after UV irradiation compared with matched controls.

The first case has been described by Pearson et al (1970). Meiosis proceeds normally up to and including the pachytene stage of first meiotic prophase, the chromosomes pairing normally. At diakinesis the number of chiasma averaged nine per cell compared with the normal value of 45-54. The remaining bivalents appeared to have fallen apart at this stage resulting in severe non-disjunction. A

second case showed a similar pattern of chromosome behaviour but the number of chiasma per cell showed a much greater range, including some with normal values.

Blood was also obtained from two individuals described by Hulten et al (1970). Both these cases resembled the previous ones, being normal until diakinesis. At this stage, univalents were again frequent although the mean chiasma counts were 39 and 41 respectively, i.e. approximately a 25% reduction compared with normal.

RESULTS

In each of these experiments induced DNA synthesis has been estimated autoradiographically by ^3H -thymidine uptake over a 2-3 hour period following exposure of lymphocytes to UV light. The potential "repair-deficient" mutant has, in each case been compared with one or two controls matched for age and sex, and a 5-10ml blood sample taken from each person. White blood cell separation, UV irradiation and subsequent incubation in medium containing ^3H -thymidine is described in Chapter II. Following fixation of the cells in 3:1 alcohol/acetic acid 2 or 3 slides were made from each sample, stained with acetic orcein (1%) and prepared for autoradiography. Grain counts over 100 lymphocyte nuclei per slide (usually two per individual) were then estimated. Fig. V 1 shows cells typical of those scored, the lymphocytes being easily distinguishable from the polymorphonuclear leucocytes.

Variation among "Normal" Individuals

In order to test the reliability of the technique as a means of comparing unscheduled DNA synthesis in different individuals, two experiments were carried out on samples from "normal" healthy

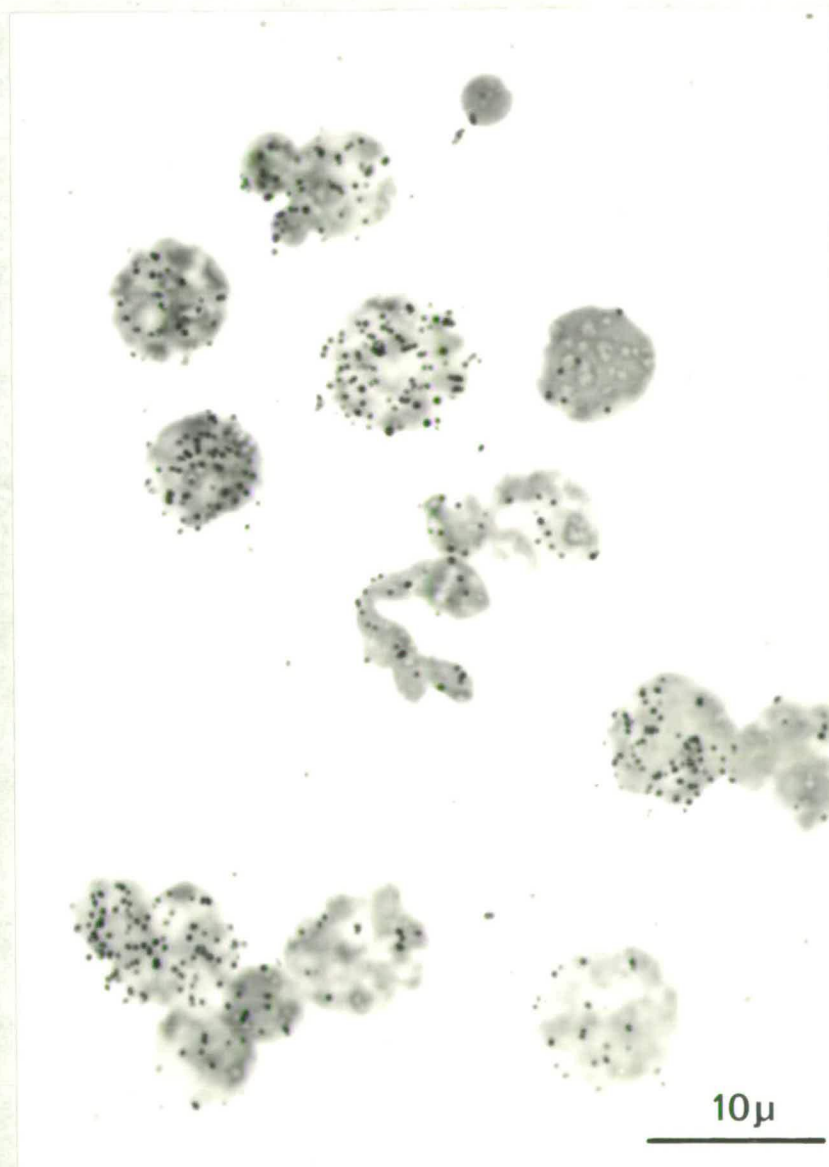


 Fig. V 1 An autoradiogram of leucocytes exposed to UV light and incubated in medium containing ^3H -thymidine. The slides are stained in orcein, a nuclear stain, so that the round nuclei of the lymphocytes are easily distinguishable from the lobed nuclei of the polymorphonuclear leucocytes.

individuals. Initially, 4 females of the same age were compared and the results are shown in table VI. The mean grain counts for 200 UV-irradiated (2800 ergs/mm^2) cells from each person (100 cells on each of 2 slides) are within the range 8.1 - 9.4, indicating that a limited range of values would be expected among control individuals matched for age and sex, and that any difference between these and the individuals of interest might be a true reflection of differences in "repair" capacities.

These original control results were obtained shortly before blood samples were received from the rare "mutants" (Bloom's syndrome from Dr German, New York; an achiasmate from Dr Pearson, Oxford; and Xeroderma pigmentosum from Dr Johnson, Aberdeen). As a consequence of some of the results obtained in these studies, further work on cells from "normal" individuals was thought to be desirable. In these subsequent studies 25ml of blood was taken from each of 5 males (aged 23 to 30) and divided into 5 samples. Each of the 25 samples was treated in a similar manner, the white cells being exposed to 700 ergs/mm^2 of UV light, incubated in ^3H -thymidine for 3 hours and slides made from each. Once the autoradiograms had been made, the slides were coded and 50 cells from one slide from each sample were scored so that results from 5 replicates from each person were obtained. The results in table V 2 are the overall mean values for each person, estimates from the 5 samples (250 cells) being pooled. 95% confidence limits show that in many cases, if pairs of individuals are compared, there is no significant difference between the means. However, between the highest (24.7) and lowest (15.4) grain count values this appears to be highly significant. This is also suggested

Table V 1 Mean grain counts over lymphocytes from 4 "normal" individuals.² Half of each sample was irradiated with 2800 ergs/mm² of UV light and the cells incubated in medium containing ³H-thymidine for 3 hours.

	Age	Mean grain count (unirradiated)	S.E.	Mean grain count (2800 ergs/mm ²)	S.E.
C.C.	22	0.6	0.64	9.4	0.46
L.Y.	22	0.5	0.54	9.4	0.53
J.C.	23	0.3	0.44	8.7	0.57
I.N.	25	0.5	0.52	8.1	0.44

Table V 2 Mean grain counts and 95% confidence limits for lymphocytes from 5 "normal" individuals. In each case the mean has been estimated from 5 samples which were irradiated with 700 ergs/mm² and incubated in medium containing ³H-thymidine for 3 hours.

Person	Mean grain count (700 ergs/mm ²)	S.E.	95% confidence limits
1	20.0	1.05	17.9-22.1
2	18.0	0.94	16.16-19.84
3	15.4	0.92	13.6-17.2
4	18.2	0.98	16.3-20.1
5	24.7	1.07	22.6-26.8

Table V 3 Result of an analysis of variance for the data in Table V 2.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
Between persons	4	11703	2925	11.4
Between samples	4	5999	1499	5.9
Corrected total	1249	317925	256	

when the data is subjected to an analysis of variance (Table V. 3). Taking the mean for each person (over 5 samples) and summing the squares of the deviations, the value in the third column is obtained. Dividing this by the degrees of freedom (4) gives the mean square and this is divided by a similar value taken from the overall mean (19.29) for the experiment, summing the squares of the deviations and again dividing by the degrees of freedom (1249). The value obtained (F) is highly significant at the 1% level (i.e. $F > 3.3$). That much of this variation is due to the size of the sample (250 cells taken as representative of the individual as a whole, especially in view of the very large range of scores: 0- >100 grains per cell) is shown by comparing different samples from the same person in the same way. Each sample from the same person was labelled a-e and the slides scored in batches of 5, one from each person, in a random fashion. The overall mean grain count for each slide was then used to calculate the squares of the deviations in a manner similar to that for each person. This was repeated for each set of slides and the results summed, and divided by the degrees of freedom (4) and the overall mean square as before. The value of F obtained (5.9) is again highly significant at the 1% level.

This difference in the levels of labelling between the individuals is also reflected in the distribution of grains shown in Fig. V. 2. This is particularly evident from the numbers of unlabelled cells (0-4 grains per cell), being 19% for a mean of 15 (person 3) and 8% for a mean of 24 (person 5). Plotted on the same graphs are the expected distributions of grains for a Poisson distribution about the same mean. It is clear that there is a large excess of these

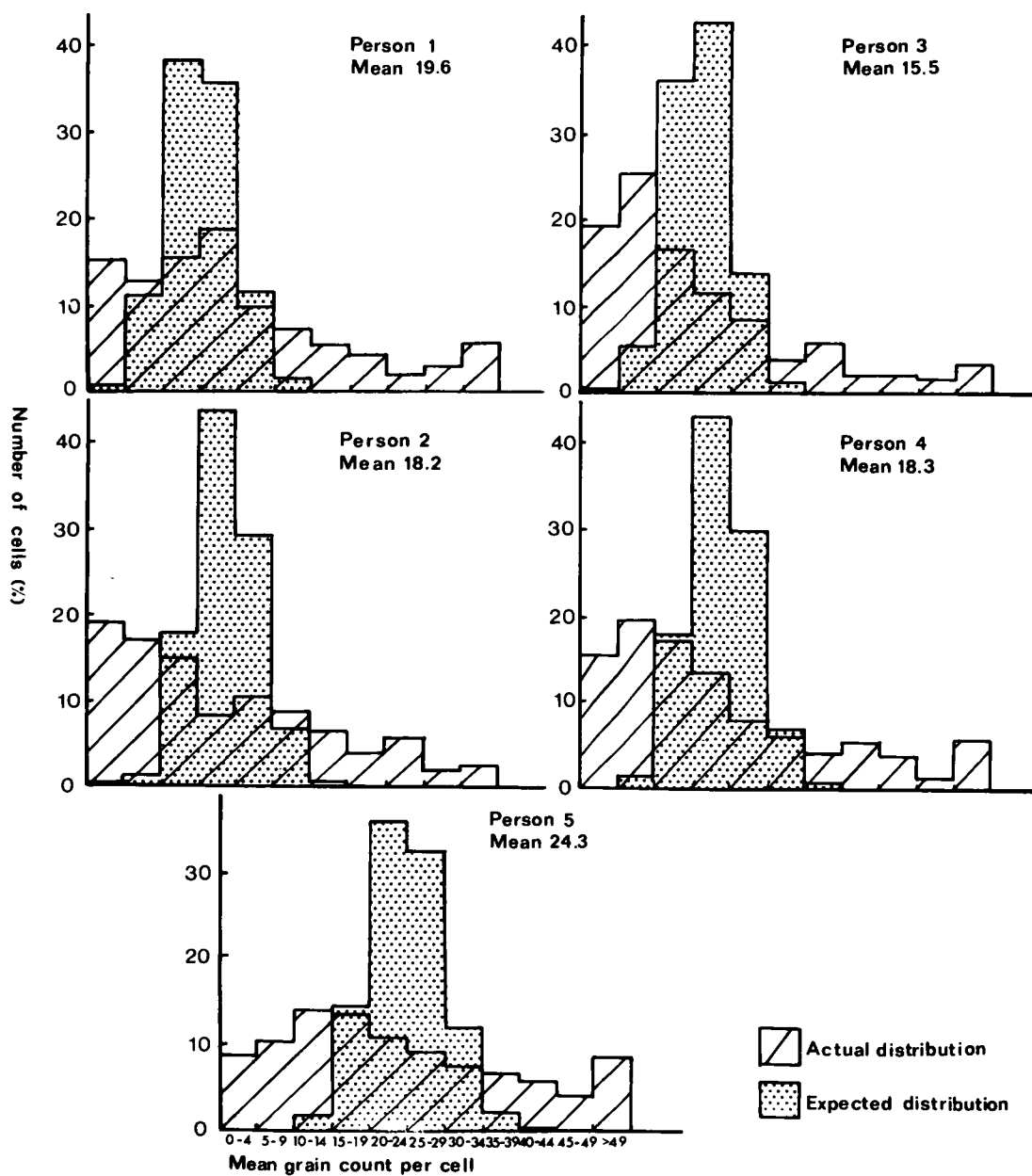


Fig. V 2 The distribution of grains over the nuclei of lymphocytes from 5 "normal" individuals. The cells were exposed to 700 ergs/mm^2 of UV light and incubated in ^3H -thymidine for 3 hours. Plotted on the same axis is the expected distribution of grains in each case for a Poisson distribution about the same mean.

unlabelled cells and their significance in relation to variations in the overall levels of synthesis is considered in the Discussion.

2. Bloom's Syndrome

The results for the comparison of the six-year old male Bloom's patient with two different controls are shown in Fig. V 3 and Tables V 4 and V 5. In the first experiment the control was also a six-year old boy, and following irradiation with 2800 ergs/mm^2 of UV light the cells (including unirradiated controls), were incubated for three hours in medium containing 20% serum from the same blood i.e. Bloom's cells in Bloom's serum and normal cells in their own serum. The graph shows that in both irradiated and unirradiated samples there is a slightly elevated mean grain count in the case of the Bloom's cells. Taking values of t for ∞ degrees of freedom (at least 200 cells per person were scored in all experiments), 95% and 99% confidence limits for the means are shown:

	95% limits	99% limits
Normal control	1.7-2.3	1.6-2.4
Bloom's control	2.9-4.0	2.7-4.2
Normal irradiated	9.1-12.3	8.7-12.7
Bloom's irradiated	12.1-15.5	11.8-15.8

In the case of the unirradiated sample this difference is significant at the 99% level, although in the irradiated samples it is barely so at 95% limits. Table V 4 shows the distribution of grains and again, approximately 10% more Bloom's than normal cells have more than 5 grains per cell and in the irradiated sample these cells are amongst the most heavily labelled (> 39 grains per cell).

In the second experiment the control was 12 years old and all the cells were incubated in the same serum i.e. foetal calf, as in all

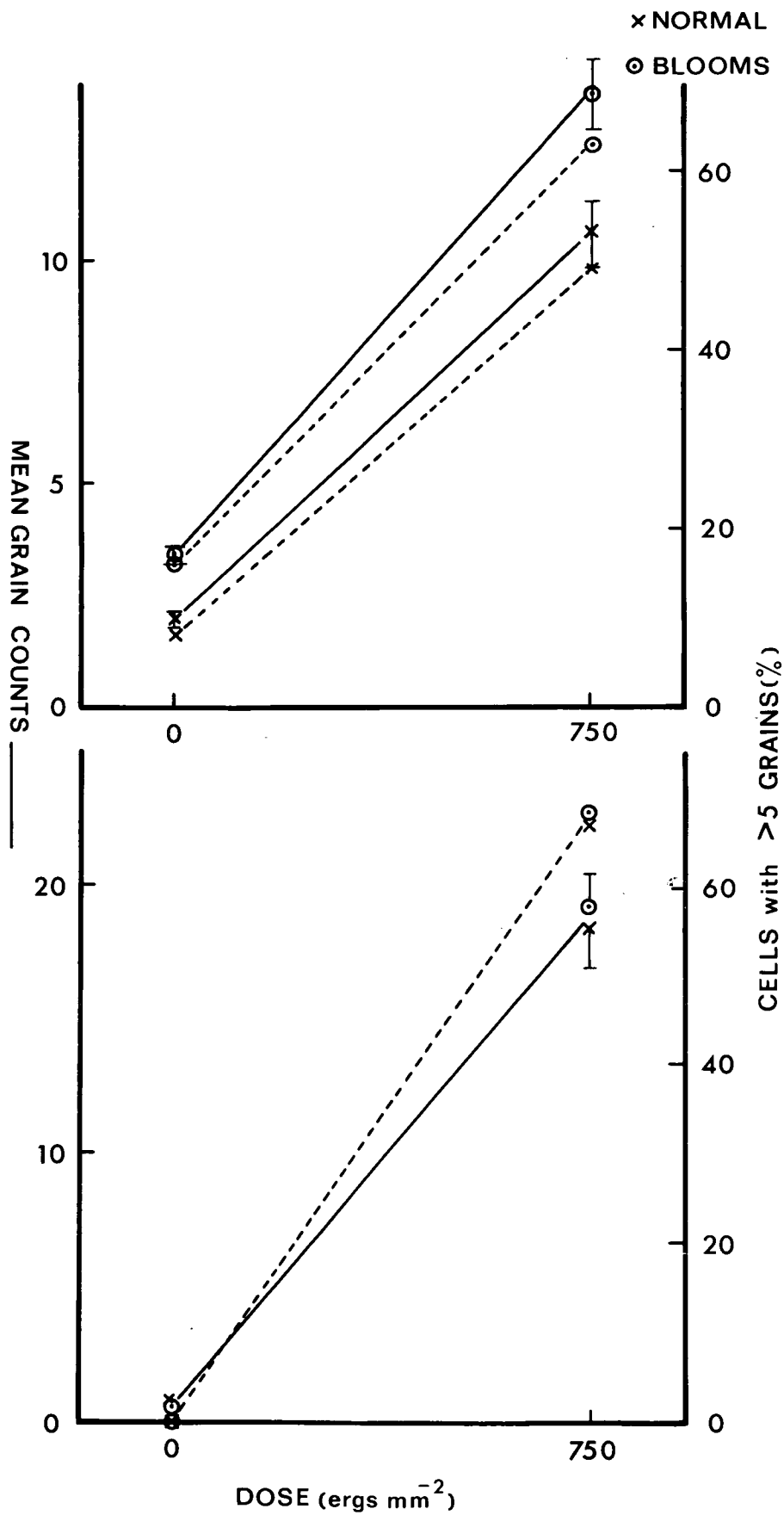


Fig. V 3 The mean grain counts over lymphocyte nuclei following exposure of the cells to UV light and incubation in ^3H -thymidine. In the upper fig. the cells from the Bloom's patient and the "normal" control were each incubated in medium containing their own serum. In the lower fig. a second control was used and both samples of cells incubated in bovine serum.

Table V 4 The distribution of grains over normal and Bloom's cells exposed to 2800 ergs/mm² of UV light and incubated in medium containing ³H-thymidine for 3 hours.

	<u>Dose</u> (ergs/mm ²)	<u>Mean grain count per cell</u>								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control	0	175	22	3						
Blooms	0	135	50	14	1					
Control	2800	94	31	16	8	7	6	9	4	25
Blooms	2800	69	30	19	12	9	5	5	8	43

Table V 5 The distribution of grains over normal and Bloom's cells exposed to 2800 ergs/mm² of UV light and incubated in medium containing ³H-thymidine for 3 hours. The result of treating the slides with DNase is also shown.

	<u>Dose</u> (ergs/mm ²)	<u>DNase</u>	<u>Mean grain count per cell</u>								
			0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control	0	-	194	6							
		+	200								
Blooms	0	-	199	1							
		+	200								
Control	2800	-	56	34	24	25	9	4	4	0	44
		+	200								
Blooms	2800	-	60	33	21	18	9	8	3	3	45
		+	200								

subsequent experiments. In this case mean grain counts and percentage labelled cells are almost identical (see Fig. V 3 and Table V 5). Part of each slide was treated with DNase as described in the materials and methods section and the results in the table show that all the grains are due to thymidine incorporated into DNA.

3. Fanconi's aplastic anaemia

In all, four cases have been considered, two of which are brothers. These are shown in Fig. V 4 and V 5 and Tables V 6 and V 7. In the first case the (heterozygous) parents, and two younger, apparently normal, children are also considered. Two normal controls were used, one the same age as the parents and the other aged 6 years to match the children who were 9 (A), 6 (B), $4\frac{1}{2}$ (C), and $2\frac{1}{2}$ (D) years old. Fig. V 4 shows the mean grain counts following 2800 ergs/mm^2 , and, where there was sufficient blood, 5600 ergs/mm^2 as well. Estimates for the unirradiated control samples are also shown in this fig. and it is evident that there is no difference between Fanconi and normal cells, the mean grain counts each being between 0.5 and 0.9. The most striking thing about these results is not the difference between irradiated Fanconi and normal cells, but, with one exception, the consistently higher level of grains in the cells from the children compared with those from adults. The 99% confidence limits for the overall means are 10.2-12.4 and 5.8-8.6 with the number of labelled cells averaging 60 and 40% respectively. Confidence limits are shown, as before, for the Fanconi children (pooled) compared with their control, and the parents (pooled) at 2800 ergs/mm^2 and the father at 5600 ergs/mm^2 compared with theirs:

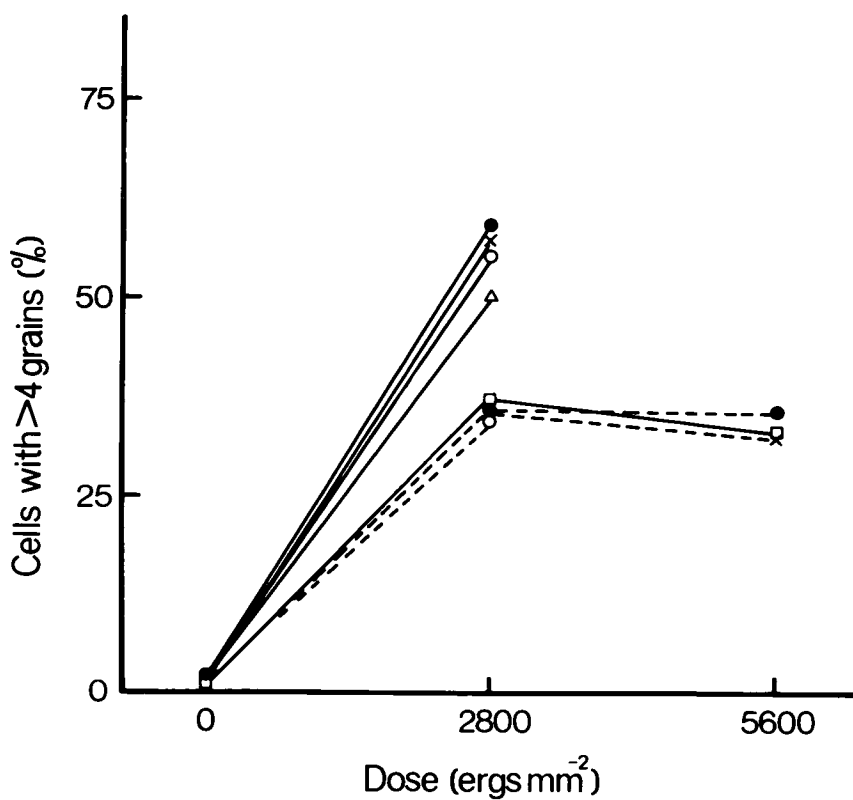


Fig. V 4 The mean grain counts (upper fig.) over lymphocytes from 2 Fanconi children (A and B), their sibs (C and D) and parents compared with controls matched for age. The cells were exposed to UV light and incubated in ^3H -thymidine for 3 hours. The number of labelled cells in each case is shown in the lower fig.

N.B. Unlike the other figs. dotted lines are used to distinguish the adults from the children.

Table V 6

The distribution of grains over normal and Fanconi cells exposed to 2800 & 5600 ergs/mm² of UV light and incubated in medium containing ³H-thymidine for 3 hours.

	Dose (ergs/mm ²)	Mean grain count per cell								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control	0	195	5							
Mother	0	197	2	1						
Father	0	196	3	1						
Control (Child)	0	200								
Child A	0	199	1							
Child B	0	197	3							
Child C	0	197	3							
Child D	0	200								
Control	2800	130	40	15	4	3	4	1	1	2
	5600	134	37	13	4	3	2	2	2	3
Mother	2800	132	29	8	9	7	2	1	4	8
Father	2800	119	26	9	13	5	8	5	3	12
	5600	130	29	13	1	3	3	4	3	14
Control (child)	2800	86	53	17	5	9	5	5	6	14
Child A	2800	89	32	15	10	12	11	14	6	11
Child B	2800	82	36	13	11	10	11	11	4	22
Child C	2800	125	30	15	4	8	2	2	1	13
	5600	133	23	13	7	4	6	3	8	3
Child D	2800	101	33	15	9	9	7	6	2	18

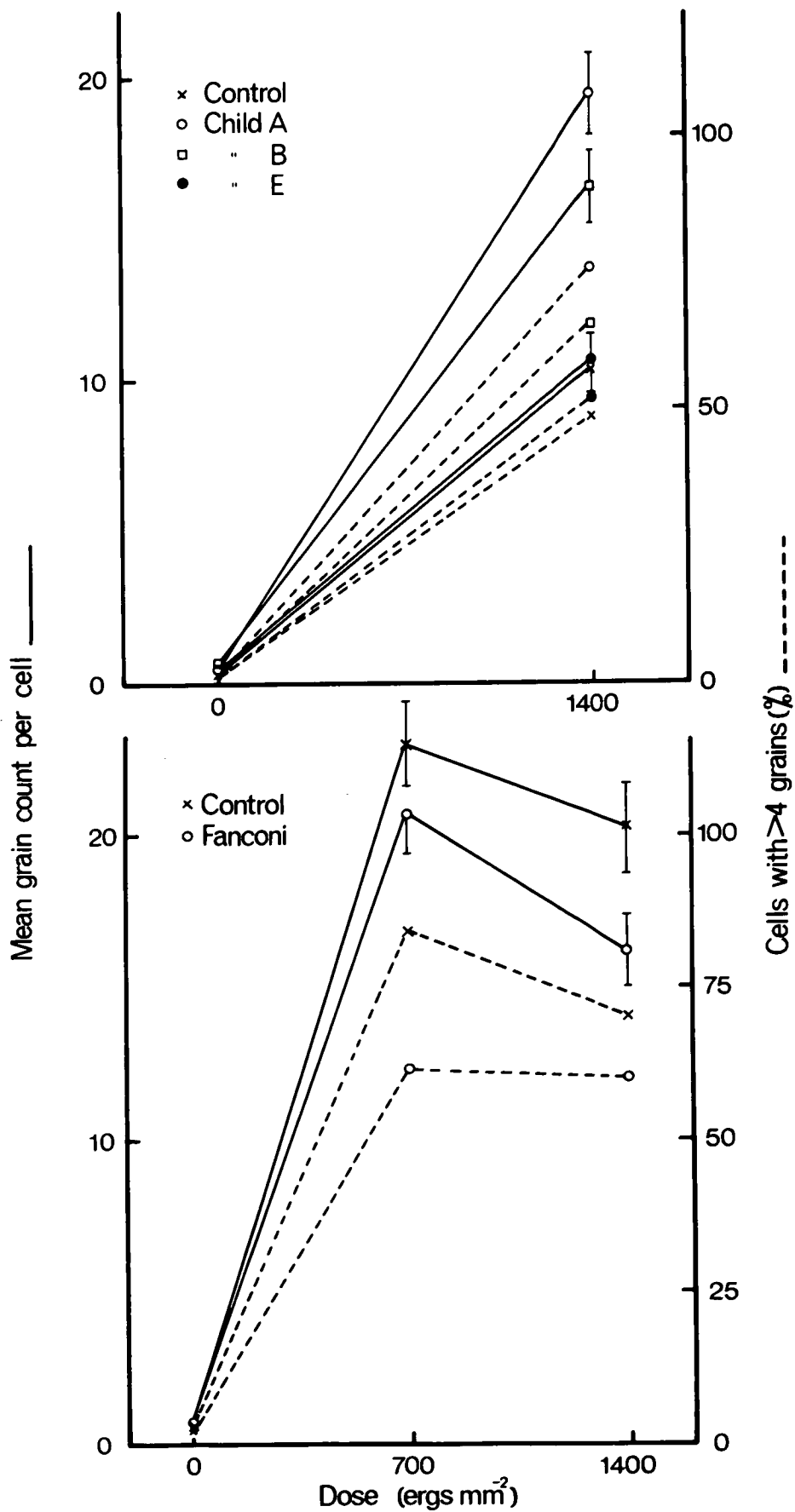


Fig. V 5 The mean grain counts over the nuclei of Fanconi and normal lymphocytes exposed to UV light and incubated in ^3H -thymidine for 3 hours. In the upper fig. the children (A and B) are the same as in fig. V 4 but compared with a different control. 2 other cases are also shown.

Table V 7 The distribution of grains over normal and Fanconi cells exposed to 1400 ergs/mm² of UV light and incubated in medium containing ³H-thymidine for 3 hours. Child A and child B are the same as in Table V 6.

	Dose (ergs/mm ²)	Mean grain count per cell								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control	0	198	2							
Child A	0	198	2							
Child B	0	198	2							
Child E	0	198	2							
Control	1400	102	19	18	16	17	7	10	4	7
Child A	1400	51	37	21	16	7	13	7	11	37
B	1400	69	39	10	13	14	8	13	7	27
E	1400	95	33	22	8	11	8	9	4	9

Table V 8 The distribution of grain over normal and Fanconi cells exposed to 700 and 1400 ergs/mm² of UV light and incubated in medium containing ³H-thymidine for 3 hours.

	Dose (ergs/mm ²)	Mean grain count per cell								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control	0	198	2							
Fanconi	0	197	3							
Control	700	33	36	24	17	18	7	9	10	46
Fanconi	700	57	21	15	12	20	14	9	13	39
Control	1400	59	29	23	12	5	13	10	14	35
Fanconi	1400	81	21	17	14	13	8	11	11	24

	95% limits	99% limits
Fanconi children (A and B)	11.8-14.8	11.5-15.1
Control (child)	9.2-13.0	8.7-13.5
Parents (2800 ergs)	6.9-9.5	6.7- 9.7
Control (2800 ergs)	4.2- 6.4	4.0- 6.6
Father (5600 ergs)	6.3-10.1	5.9-10.5
Control (5600 ergs)	4.2- 6.8	4.0- 7.0

Although both the Fanconi children have higher mean grain counts than their control, these differences do not appear to be significant in these data. However the heterozygous parents do show a significant increase at 2800 ergs/mm² although this is not confirmed at 5600 ergs/mm².

When these same two children (A and B) were compared with a different control (a nine year old male) each was significantly different from it at the 99% confidence limits: 16.4-22.4 and 13.4-19.2 compared with 8.3-12.5. A third patient (E) aged nine years, from a different family, but tested at the same time, showed the same uptake of label as the control (see Fig. V 5). The distribution of grains shown in Table V 7 confirms these results, children A and B showing many more cells with more than 40 grains than the control or child E.

The results from a fourth case are shown in Fig. V 5 and Table V 8. Both show that the level of labelling is very similar to that in the control and 95% confidence limits for the mean grain counts are 21.2-25.8 (control) and 19.1-23.3 (Fanconi) at 700 ergs/mm² and 17.4-23.0 (control) and 13.7-18.7 (Fanconi) at 1400 ergs/mm².

4. Keroderma pigmentosum (Xp)

This patient was compared with four different controls in two separate experiments (see Fig. V 6 and Tables V 9 and V 10). In the

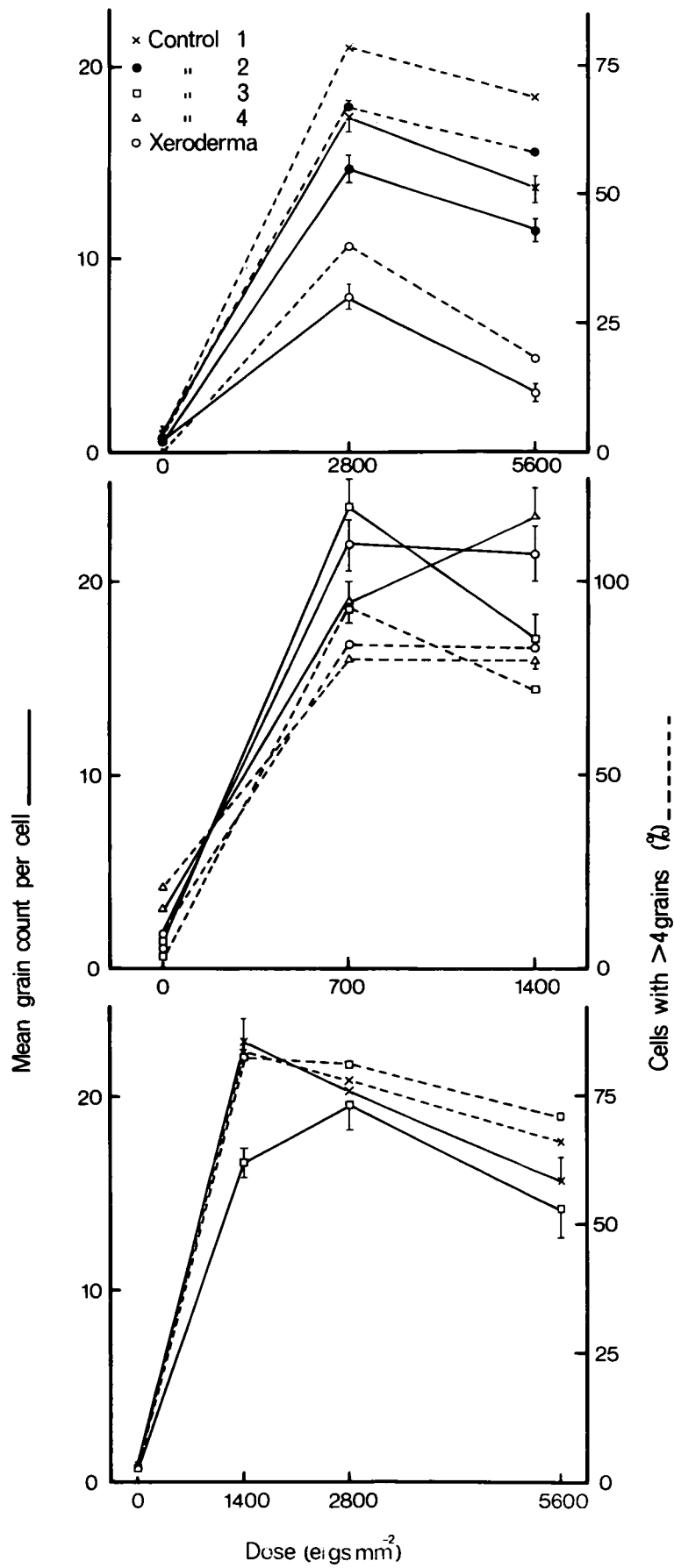


Fig. V 6 The mean grain count over the nuclei of Xeroderma and normal lymphocytes exposed to various doses of UV light and incubated in medium containing ^3H -thymidine for 3 hours. In the top fig. the Xp cells were compared with 2 controls at high doses (2800 and 5600 ergs/mm²), and in the centre with 2 different controls at lower doses (700 and 1400 ergs/mm²). The bottom fig. shows a comparison of cells from one control from each of these experiments.

Table V 9 The distribution of grains over Xeroderma and normal cells exposed to 2800 and 5600 ergs/mm² of UV light and incubated in medium containing ³H-thymidine for 3 hours.

	Dose (ergs/mm ²)	Mean grain count per cell								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control 1	0	193	7							
Control 2	0	199	1							
Xp	0	199	1							
Control 1	2800	38	48	27	19	15	19	12	12	10
Control 2	2800	63	31	35	17	14	8	7	8	17
Xp	2800	111	29	21	11	12	7	4	2	3
Control 1	5600	60	49	36	20	12	14	3	3	3
Control 2	5600	74	40	26	15	16	11	12	3	3
Xp	5600	153	27	11	3	3	0	0	1	2

Table V 10 The distribution of grain over cells from the same Xp patient as in Table V 9 but compared with different controls. The cells were irradiated with 700 and 1400 ergs/mm² and incubated in ³H-thymidine for 3 hours.

	Dose (ergs/mm ²)	Mean grain count per cell								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control 3	0	192	8							
Control 4	0	158	42							
Xp	0	191	9							
Control 3	700	16	36	26	34	21	7	4	13	43
Control 4	700	42	25	20	28	19	17	19	13	17
Xp	700	30	32	37	20	13	9	17	9	33
Control 3	1400	58	33	25	28	20	8	5	4	19
Control 4	1400	40	32	16	16	12	17	11	14	42
Xp	1400	35	36	37	21	16	12	5	6	38

Table V 11 A comparison of grain densities over cells from the controls used in the experiments shown in Tables V 9 and V 10. A range of doses was used and the cells incubated as before.

	<u>Dose</u> (ergs/mm ²)	<u>Mean grain count per cell</u>								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control 1	0	199	1							
Control 3	0	198	1			1				
1	1400	36	28	36	19	8	8	8	10	47
3	1400	34	53	30	24	22	7	4	7	19
1	2800	44	41	21	16	12	9	7	9	41
3	2800	38	42	35	28	9	6	8	6	32
1	5600	68	38	21	16	7	9	9	6	26
3	5600	58	46	36	12	16	4	6	4	18

first case dose levels of 2800 and 5600 ergs/mm² were used and at both levels a very significant difference between Xp cells and each control was found; 99% confidence limits being 6.3-9.7 compared with 15.5-19.3 and 12.7-16.7 at 2800 ergs/mm² and 2.2-4.0 compared with 11.8-15.6 and 10.0-12.8 at 5600 ergs/mm². This is also reflected in the number of labelled cells (>5 grains/cell).

In the second experiment dose levels of 700 and 1400 ergs/mm² were used and the mean grain count for the Xp cells falls between that of the controls at both doses (see Fig. V 6). It was assumed that the difference between the results of the two experiments was due to one of two factors, namely, differences in the responses of the controls, or, a dose effect. The former was eliminated by comparing a control from each experiment (Fig. V 6, Table V 11). The slight difference at 1400 ergs/mm² would not account for the differences observed in the first experiment and comparing the overall mean for the two controls 95% confidence limits are 15.4-18.2 and 18.2-21.4 respectively.

This would suggest that a dose effect is responsible for these results, a factor not inconsistent with the data for 2800 and 5600 ergs/mm². For the controls the level of labelling is just significantly (at the 95% confidence level) lower at the higher dose. However in the Xp cells this difference is considerable resulting in a mean grain count only 25% of that in the controls at 5600 ergs/mm² compared with 50% at the lower dose.

5. Christchurch Chromosome

H.W. who has both a Ch' chromosome and mediastinal and retro-peritoneal fibrosis has been compared with four different (normal) controls in three experiments (Fig. V 7 and Tables V 12, V 13 and V 14).

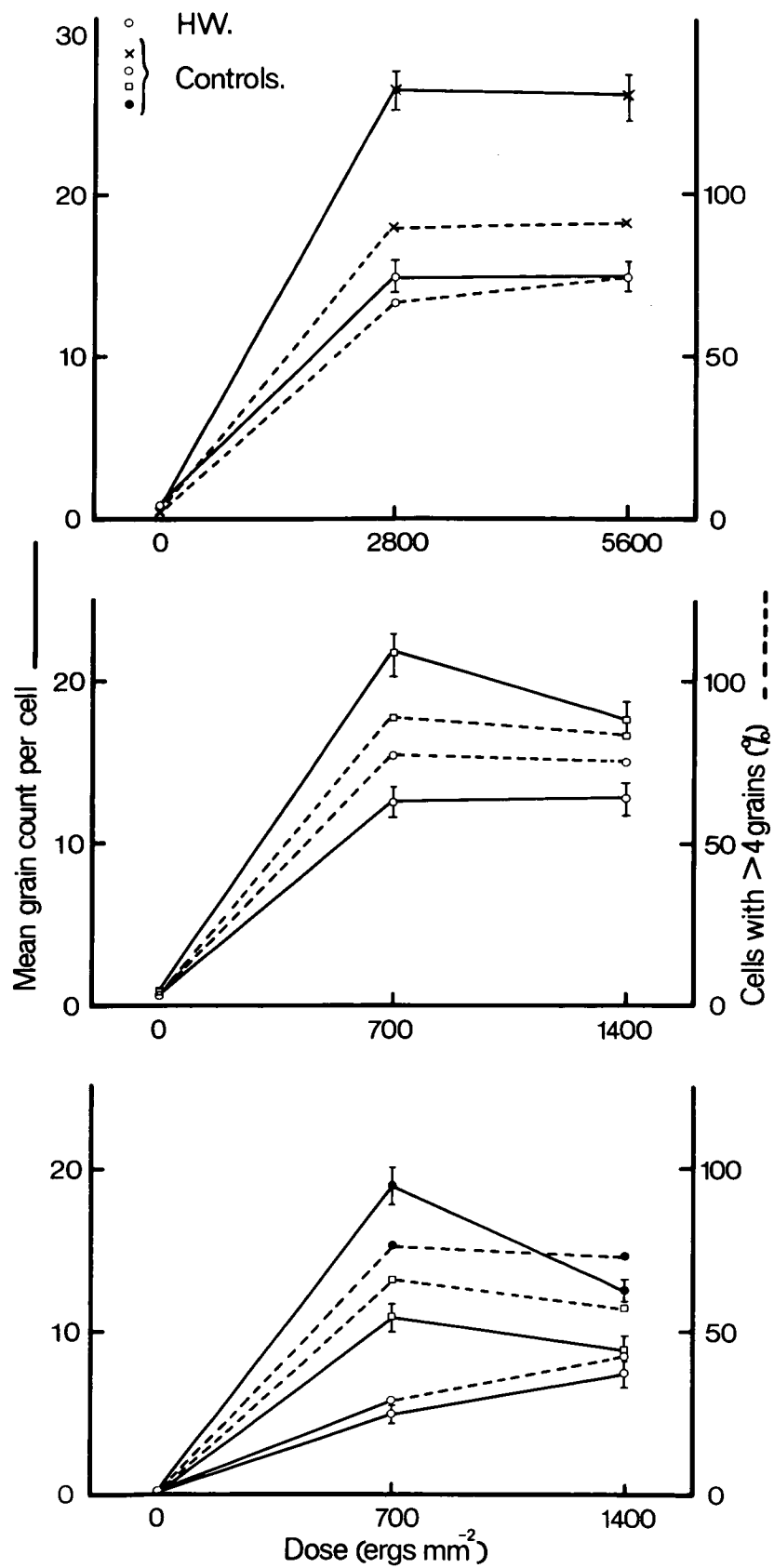


Fig. V 7 The mean grain count over nuclei of lymphocytes from an individual (H.W.) with a Christchurch chromosome and mediastinal and retroperitoneal fibrosis. The results of 3 experiments are shown, involving 3 different controls and various dose levels of UV light.

Table V 12

The distribution of grains over cells from H.W. compared with a normal control. ² The cells were exposed to 2800 and 5600 ergs/mm² UV light and incubated in ³H-thymidine for 3 hours.

	Ch'	Disease	Dose (ergs/mm ²)	Mean grain count per cell								
				0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
K.B.	-	none	0	198	2							
H.W.	+	fibrosis	0	197	3							
K.B.			2800	16	24	23	19	13	16	12	14	63
H.W.			2800	60	42	19	18	16	14	4	4	22
K.B.			5600	18	33	30	20	14	7	14	12	52
H.W.			5600	54	59	32	14	10	9	6	3	13

Table V 13

The distribution of grains over cells from H.W. compared with a different control. Lower UV doses were used, but other experimental details as in Table V 12.

	Ch'	Disease	Dose (ergs/mm ²)	Mean grain count per cell								
				0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
M.N.	-	none	0	197	3							
H.W.	+	fibrosis	0	198	2							
M.N.			700	22	27	29	23	20	22	21	13	23
H.W.			700	46	55	48	13	13	5	7	3	10
M.N.			1400	34	34	34	28	18	11	14	9	18
H.W.			1400	49	57	37	17	5	12	6	8	9

Table V 14 The distribution of grains over cells from H.W. compared with 2 different controls, (M.N. as in Table V 13).² The cells were exposed to 700 and 1400 ergs/mm² UV light and incubated in ³H-thymidine as before.

	Ch ¹	Disease	Dose (ergs/mm ²)	Mean grain count per cell								
				0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
E.K.	-	none	0	200								
M.N.	-	none	0	200								
H.W.	+	fibrosis	0	199	1							
E.K.			700	45	24	25	29	15	11	16	5	30
M.N.			700	68	44	28	21	14	12	1	7	4
H.W.			700	142	26	18	5	4	2	3	2	0
E.K.			1400	53	50	25	27	19	10	4	3	2
M.N.			1400	83	45	22	17	13	9	3	3	5
H.W.			1400	115	44	12	6	7	3	3	3	7

99% confidence limits for the mean grain counts are shown, the data for the two controls in the third experiment being pooled:

Experiment	Dose	H.W.	Control
1	2800 ergs/mm ²	12.8-17.2	23.9-29.5
	5600 ergs/mm ²	12.6-17.2	22.8-29.4
2	700 ergs/mm ²	10.6-14.6	19.2-24.4
	1400 ergs/mm ²	10.7-14.9	15.4-20.0
3	700 ergs/mm ²	3.7- 6.1	13.3-16.7
	1400 ergs/mm ²	5.5- 9.3	9.8-12.6

In all cases (covering a wide dose range) the difference is highly significant and again the reduction is at least partially due to a reduction in the number of labelled cells. In the first two experiments more than twice as many cells have > 39 grains in the controls (see Tables V 12 and V 13).

In Fig. V 8 individuals with the same fibrosis (E.H.) and another example of a Christchurch chromosome (R.B.) have been compared with normal controls. The confidence limits for E.H. and its control are:

	95% limits	99% limits
E.H. 700 ergs/mm ²	12.8-16.8	12.5-17.1
Normal 700 ergs/mm ²	16.5-19.5	16.1-19.9
E.H. 1400 ergs/mm ²	11.6-14.8	11.2-15.2
Normal 1400 ergs/mm ²	16.7-21.1	16.3-21.5

Although the reduction is significant at the higher dose it is not at 700 ergs/mm² even at the 95% limits. It is therefore likely that

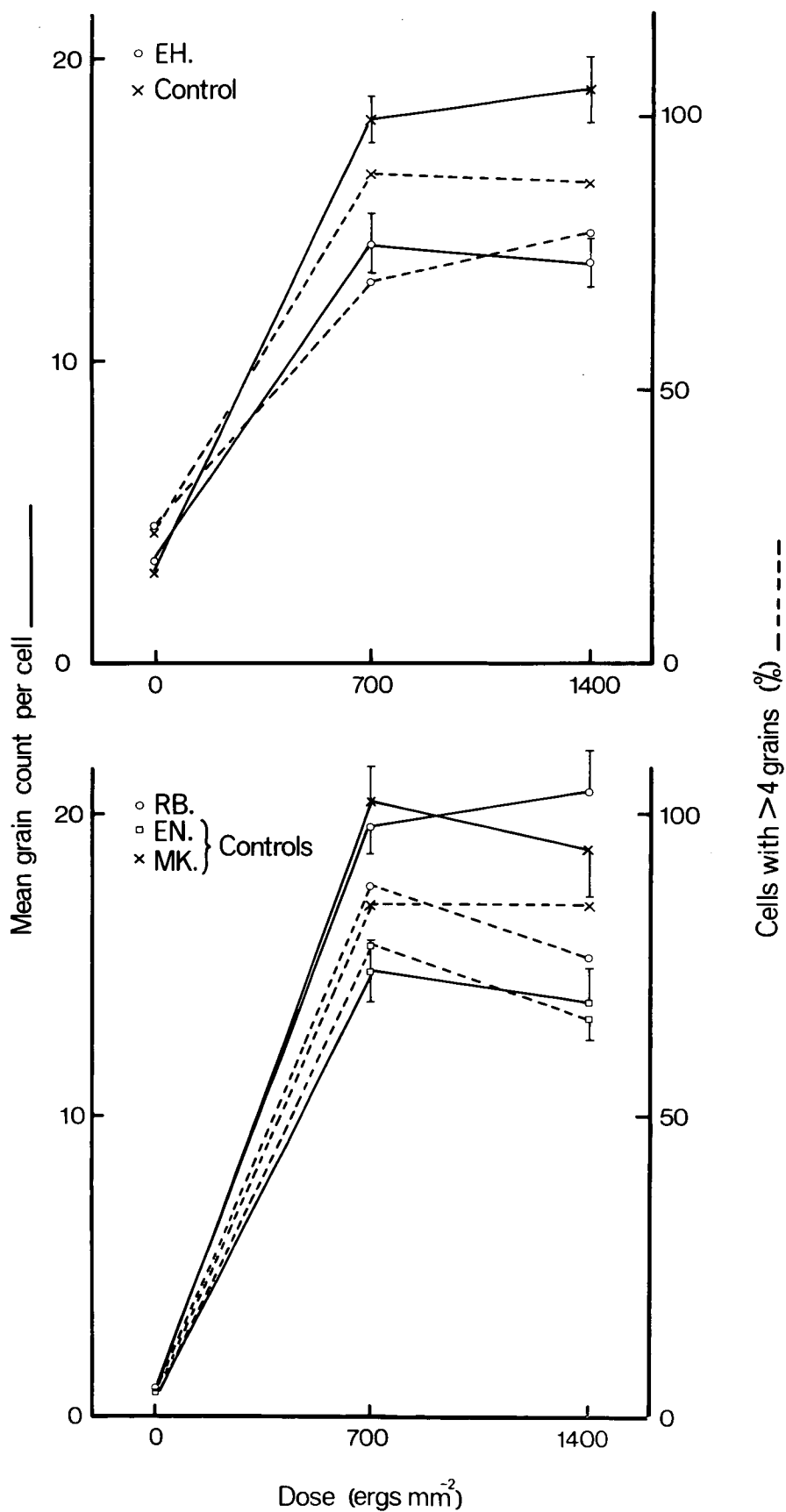


Fig. V 8 In the upper fig. the mean grain count over the nuclei of lymphocytes from a normal individual are compared with those from a patient with mediastinal and retroperitoneal fibrosis. This is the same complaint as that of H.W. (see fig. V 7) and the 2 results should be compared. In the lower fig. the results for cells from R.B. (an individual with a Ch' chromosome and undergoing radiotherapy for bladder cancer) are compared with those from a "normal" control and with those from a control also undergoing radiotherapy (M.K.). In each case lymphocytes were exposed to UV light and incubated in ^3H -thymidine for 3 hours.

Table V 15 The distribution of grains over cells from E.H. compared with a normal control.² The cells were exposed to 700 and 1400 ergs/mm² and incubated in ³H-thymidine for 3 hours.

	Ch'	Disease	Dose (ergs/mm ²)	Mean grain count per cell								
				0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
H.E.	-	none	0	152	36	8	2	2				
E.H.	-	fibrosis	0	151	28	11	3	3	3	1		
H.E.			700	20	30	37	38	20	24	16	5	10
E.H.			700	61	33	27	14	19	10	10	14	12
H.E.			1400	25	42	31	29	16	15	9	9	24
E.H.			1400	44	59	33	25	10	10	8	5	6

Table V 16 The distribution of grains over cells from R.B. compared with a normal control and also a control undergoing radiotherapy (as R.B.).² The cells were exposed to 700 and 1400 ergs/mm² UV light and incubated in ³H-thymidine for 3 hours.

	Ch'	Disease	Radio- therapy	Dose (ergs/mm ²)	Mean grain count per cell								
					0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
E.N.	-	none	-	0	198	2							
M.K.	-	Ca brest	+	0	196	4							
R.B.	+	Ca bladder	+	0	198	2							
E.N.				700	45	57	37	18	8	8	7	3	17
M.K.				700	31	47	29	12	14	10	12	17	28
R.B.				700	24	53	41	14	20	7	4	3	32
E.N.				1400	67	45	23	15	13	11	5	3	18
M.K.				1400	29	46	33	20	13	12	8	10	29
R.B.				1400	28	44	30	16	12	15	12	8	35

this difference can be accounted for by the variation among normal individuals and that the reduction shown for H.W. is not associated with mediastinal and retroperitoneal fibrosis.

R.B. was compared with two controls. All three individuals had a similar number of labelled cells and by pooling the data for the controls 99% confidence limits for the means shows no significant difference:

	700 ergs/mm ²	1400 ergs/mm ²
R.B.	16.3-22.9	18.1-24.7
Controls	15.5-19.9	14.4-18.4

6. Achiasmate Males

The results for the four desynaptic individuals studied are shown in Fig. V 9 and V 10 and in Tables V 17, V 18 and V 19. In the first case, the individual with a mean chiasma count of 9, there is a considerable reduction in the mean grain count compared with the control, the level of labelling being approximately 55 and 60% for the two doses with 99% confidence limits for the means of 17.0-23.8 and 12.1-18.3 compared with 32.0-41.2 and 20.7-27.9. A similar picture is evident from the distribution of grains, approximately twice as many control cells have > 39 grains (Table V 17).

The other three individuals (in which the chiasma counts were about 40 per cell) show no similar reduction compared with the controls used. One of them (Fig. V 9 and Table V 18) was compared with two individuals of the same age and has a very similar distribution of grains. Values for the mean grain counts show that the achiasmate has a very similar uptake of label compared with one control and as

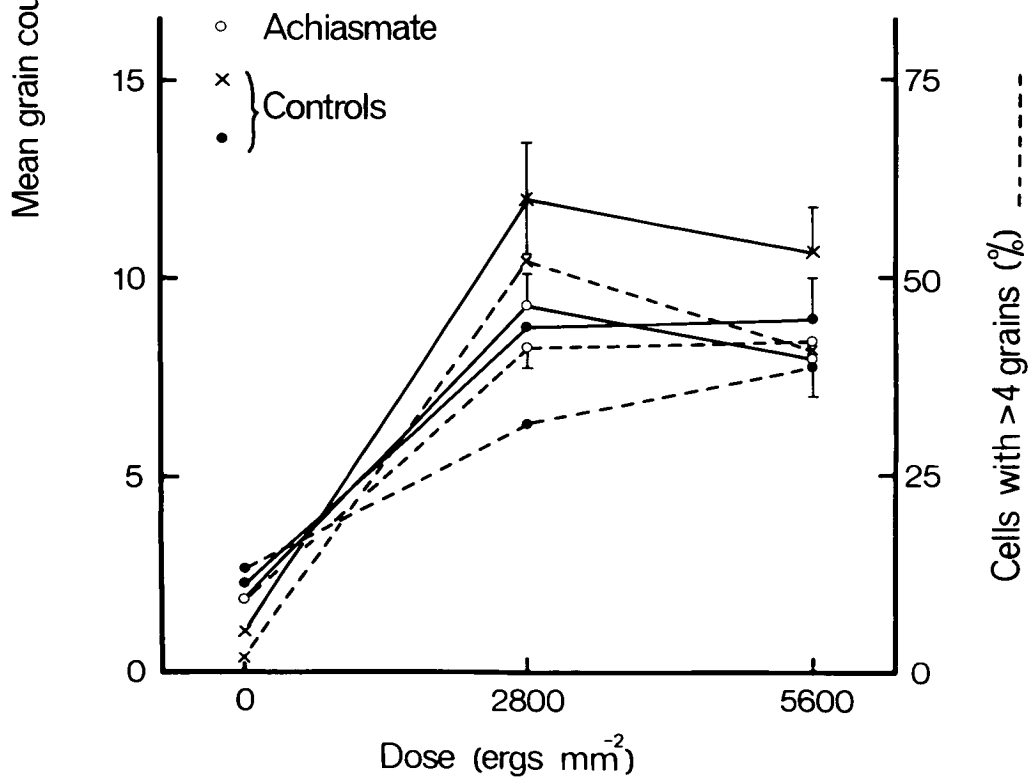
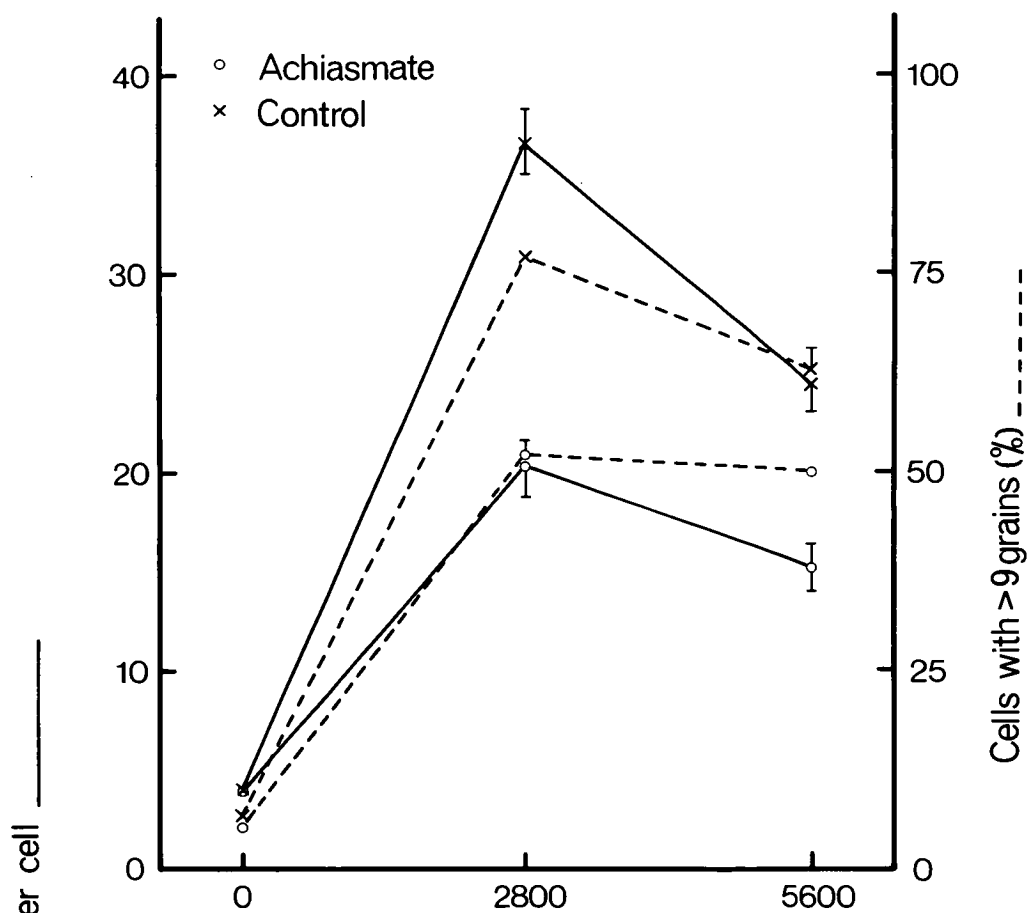


Fig. V 9 The mean grain counts over UV-irradiated lymphocytes from individuals with reduced chiasma counts at meiosis. The results in the upper fig. are for an individual with a mean chiasma count of 9; the individual represented in the lower fig. had a range of values including some normal cells.

Table V 17 The distribution of grains over cells from an individual with a mean chiasma count at meiosis of 9. These cells together with those from a control individual were exposed to 2800 and 5600 ergs/mm² of UV light and incubated in ³H-thymidine.

	Dose (ergs/mm ²)	Mean grain count per cell								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control	0	138	57	5						
Achiasmate	0	140	52	8						
Control	2800	29	21	26	20	10	8	8	7	71
Achiasmate	2800	47	44	32	17	5	8	8	7	32
Control	5600	46	28	21	25	13	10	7	8	42
Achiasmate	5600	72	44	22	16	8	8	3	5	22

Table V 18 The distribution of grains over cells from an individual with a 25% reduction in chiasma count at meiosis. These cells together with those from 2 controls were irradiated with 2800 and 5600 ergs/mm² of UV light and incubated in ³H-thymidine for 3 hours.

	Dose (ergs/mm ²)	Mean grain count per cell								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control 1	0	163	30	4	3					
Control 2	0	194	5	1						
Achiasmate	0	181	15	2	2					
Control 1	2800	135	14	10	6	8	3	8	2	14
Control 2	2800	91	38	15	17	6	2	5	3	23
Achiasmate	2800	108	38	17	7	5	9	3	2	11
Control 1	5600	119	32	12	4	3	4	6	3	17
Control 2	5600	107	39	10	10	5	1	3	3	19
Achiasmate	5600	105	28	17	10	5	3	7	5	20

there is no reason to suppose that this is not within the normal range, the higher value of the other control cannot be considered significant, a fact borne out by the 95% confidence limits for the means:

	2800 ergs/mm ²	5600 ergs/mm ²
Achiasmate	7.3-11.3	5.6-10.4
Controls	6.3-11.3	6.5-11.3
	8.6-15.4	8.2-13.2

The other two cases were aged 26 (A) and 48 (B), each with a control of the same age. However this age difference does not seem to be important, the achiasmata having comparable levels of labelling with both the controls (Fig. V 10 and Table V 19).

DISCUSSION

1. General Considerations

The estimation of DNA synthesis by the incorporation of tritiated thymidine as determined by autoradiography is a widely-used technique. The experiments with DNase (Table V 5) confirm that the thymidine is in fact being incorporated into DNA, although these estimates do not take into account possible differences in the rates of precursor synthesis including such steps as phosphorylation of thymidine. As a means of screening for "repair"-deficient mutants, then, this technique has certain limitations. In addition, the significance of any difference between a potential mutant and its control depends on the variation in this character among a population of "normal" individuals. The results in Tables V 1, V 2 and V 3 suggest that this may be rather large, making it difficult to

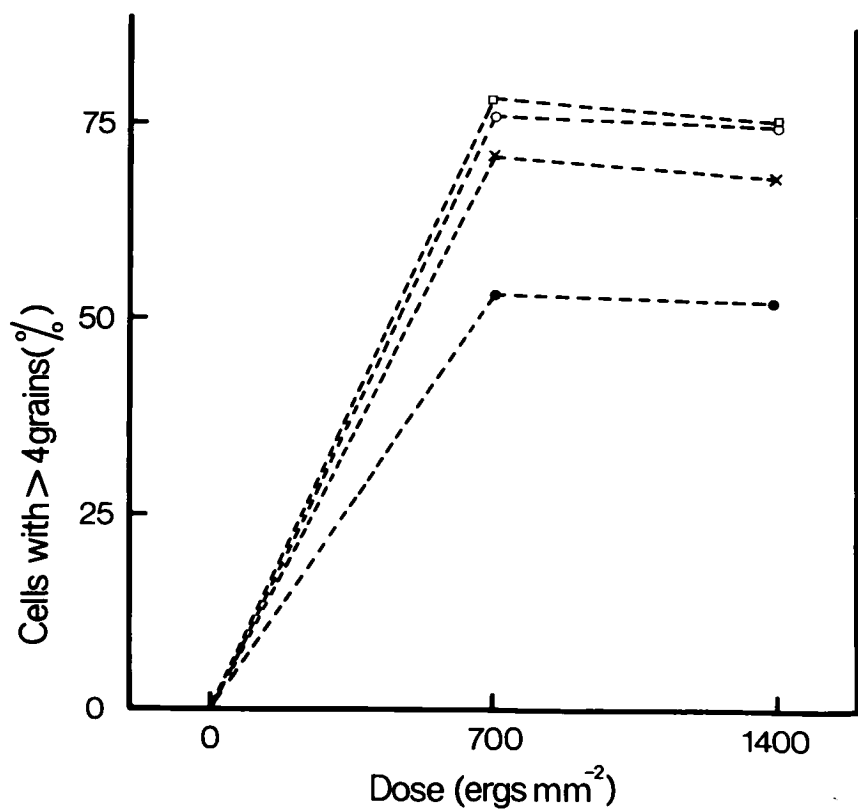
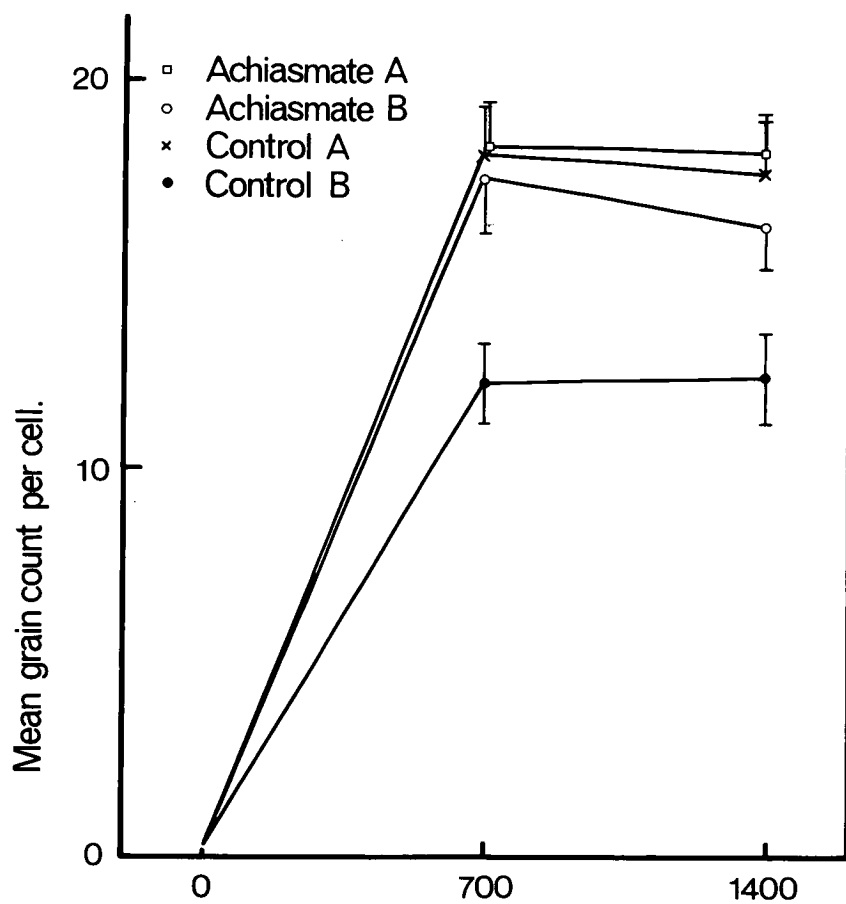


Fig. V 10 The mean grain counts over nuclei of lymphocytes from individuals with a 25% reduction in chiasma count at meiosis. Each is compared with a control of similar age, lymphocytes from each of the 4 individuals being exposed to UV irradiation and incubated in ³H-thymidine for 3 hours.

Table V 19 The distribution of grains over cells from 2 individuals each with a 25% reduction in chiasma count at meiosis. These cells, together with those from controls of similar ages were irradiated with 700 and 1400 ergs/mm² of UV light and incubated in ³H-thymidine for 3 hours.

	<u>Dose</u> (ergs/mm ²)	<u>Mean grain count per cell</u>								
		0-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39	>39
Control A	0	199	1							
Control B	0	197	2	1						
Achiasmate A	0	199	1							
Achiasmate B	0	198	2							
Control A	700	57	25	19	16	19	17	10	10	27
Control B	700	93	24	17	16	9	11	12	6	12
Achiasmate A	700	45	31	23	27	12	13	12	12	25
Achiasmate B	700	48	33	30	17	13	12	11	13	23
Control A	1400	64	19	30	16	15	6	12	7	31
Control B	1400	96	22	19	16	11	5	9	3	19
Achiasmate A	1400	60	27	22	16	15	12	8	8	32
Achiasmate B	1400	49	39	26	16	18	19	6	11	16

distinguish between small, but consistent, differences between individuals. The technique is clearly adequate to detect large deviations in the level of "repair". However, where possible 2 controls have been used and with two exceptions (see Tables V 14 and V 18) the results of these have correlated well. There is of course always the possibility that these controls have reduced synthesis for some reason. For instance, in this second example it was not possible to obtain controls in which chiasma counts had been determined. By using enough controls and repeating the experiments several times these problems could be overcome. The difficulty of obtaining blood samples meant, however, that this was not always possible.

In Fig. V 2 the distribution of grains for the 5 "normal" individuals is shown and it is evident that these reflect the variation shown by the means. This is particularly evident from the numbers of unlabelled cells which, in addition, are far in excess of that expected for a Poisson distribution. This could be due either to a shielding effect during the UV irradiation or to an inability of some of the cells to perform unscheduled DNA synthesis. The former is unlikely as the cell suspension was stirred throughout the irradiation period and is confirmed when the results are compared with those for similar experiments with X-irradiation. Here there is also a disproportionately large number of cells in the 0-4 grains/cell category (Adams 1970). The variation among individuals may therefore be due to a varying number of cells that are unable to carry out unscheduled DNA synthesis.

The importance of choosing controls of a similar age is illustrated in Table V 6 and Fig. V 4. With the exception of child C, all the

children (aged $2\frac{1}{2}$ - 9 years) show a greater uptake of label than the adults tested and when the data for each is pooled this difference is highly significant. More extensive studies would have to be carried out before generalisations on ageing and "repair" could be made, especially as Goldstein (1971) showed for fibroblasts no increase in radiosensitivity with increasing age of donor. He did however show increased sensitivity and reduced unscheduled DNA synthesis with increasing passage number, although the latter effect was not evident until this was very large (more than 56 generations).

These experiments have all been carried out with incubation times of two to three hours and there is therefore no estimation of rate of synthesis. Burk et al (1971) and Robbins & Kraemer (1972) suggest that over a sufficiently long incubation time cells from Xeroderma pigmentatum patients will incorporate as much ^3H -thymidine as normal and that reduced levels detected after three hours (Cleaver 1968) are due to a reduced rate of synthesis.

Another point to bear in mind is that only a very limited part of the repair process is being considered. Two models for the mechanism of excision repair have been postulated (Setlow 1967a) and each involves at least three steps of which the last is rejoining of the newly-synthesised region to the DNA backbone to give an intact strand. Mutations in this process would not be detected by estimations of the amount of unscheduled DNA synthesis with the result that some "repair-deficient" mutants will be undetected. Another step in the process involves degradation of the damaged regions and no attempt has been made to estimate this. A mutation at this stage could result in random excision and a net loss of genetic material as

occurs in the rec^- strain of E.coli K12 (Clark & Chamberlin 1966).

2. Bloom's Syndrome and Fanconi's Anaemia

These results are not altogether conclusive. Where differences between the control and mutant individual exist, they are small and probably within the margin of error inherent in the experiment. However, where there are differences, they consistently show an increased synthesis in the mutant as in the case of the Fanconi children A and B, who have been tested twice against different controls, and in their heterozygous parents. No increase is evident in the unirradiated samples as is the case for the Bloom's data, a small increase being evident in both irradiated and unirradiated cells when they are incubated in their own serum, but not in bovine serum. In none of the experiments are Fanconi cells (control or irradiated) incubated in their own serum.

The one thing that can be said with reasonable certainty is that the presence of spontaneous chromosome aberrations in these conditions is not due to an inability to undergo unscheduled DNA synthesis. However as mentioned earlier there is also the possibility that other stages in the repair process are defective. Perhaps excessive degradation would result in increased synthesis in an attempt to replace missing material. These results therefore give no indication of the importance of DNA repair in the rejoining of damaged chromosomes, a connection suggested by Evans (1966) and Kihlman and Hartley (1967). Wolff and Scott (1969) were also unable to correlate levels of unscheduled DNA synthesis with repair of chromosome breaks using both Chinese hamster cell lines with differing levels of unscheduled synthesis and Vicia faba in the presence and absence of caffeine.

Similarly, Shaeffer et al (1971) has demonstrated chromosomal rejoining in the absence of unscheduled DNA synthesis.

There would seem, then, that there are two possible explanations for these genetic defects associated with these conditions. Either the cells lack the necessary repair enzymes to deal with damage incurred during the normal course of events, or that the chromosomes are particularly prone to damage (possibly by viral agents, see below). The experiments reported here have been unable to resolve the first possibility, and data from other workers does little to elucidate the second.

The deterioration of Fanconi patients following viral infections has led Swift and Hirschhorn (1966) to suggest that the chromosome breakage observed in such patients could be virus-induced. The ease with which these cells are transformed with SV40 virus (Todaro et al 1966; Young 1971) also suggests that viruses could play a significant role in this syndrome. A similar deterioration following infections does not seem to occur in Bloom's syndrome (Bloom et al 1966) or Ataxia telangiectasia patients (Gropp and Flatz 1967) and the search for viruses or similar harmful agents in the serum has not been fruitful. Incubation of normal cells in serum from Bloom's (Adams 1970) or Fanconi patients (Bloom et al 1966; Perkins et al 1969) has not resulted in significant increases in aberration yields.

Swift and Hirschhorn (1966) have also suggested that a defect in the lysosomes resulting in the release of destructive enzymes could account for the chromosome damage in Fanconi's anaemia. Allison and Paton (1965) have shown that selective damage to lysosomes and release of their enzymes causes chromosome damage. Lysosomal DNase had already been shown to cause double-strand breaks (Bernardi and Sadrow 1964) and

they point out that this kind of damage would be difficult to repair. Considerable breakdown of host cell DNA following infection with herpes simplex virus is apparently due to activation of lysosomal DNase (Newton et al (1962). Perhaps the significance of virus infections in Fanconi's anaemia can be explained in this way.

The susceptibility of the chromosomes of Bloom's and Fanconi patients to radiation-induced damage has also been investigated. X-irradiation of lymphocytes has, in both cases, resulted in aberration yields not significantly different from those obtained in normal cells treated in the same manner (Adams 1970; Buckton et al 1970). However Schuler et al (1969) was able to show a greater response in Fanconi cells treated with the alkylating agent tetramethanesulfonate.

In conclusion then, it is evident that these syndromes have associated with them a defect in the mechanism responsible for maintaining the integrity of the genetic material. In view of the normal response to X-ray induced damage this mechanism may only operate under certain circumstances e.g. at certain stages in the cell cycle, or in response to specific types of damage. It is difficult to assess the importance of DNA repair in this system, but if the elevated levels are significant, it may suggest a defect in this system. In view of the high incidence of leukaemia in these patients and the already established connection between a predisposition to malignancy and reduced repair replication in Xeroderma pigmentosum cells, this would not seem to be too unlikely.

3. Xeroderma pigmentosum (Xp)

The apparent dose-dependent response of these Xp cells compared with the controls differs from any other case so far reported.

Cleaver (1968), Bootsma et al (1970) and Setlow et al (1969) all used doses of 700 ergs/mm² or less and showed reduced levels of repair in all cases. .

The fact that this individual was apparently healthy at 51 years of age indicates that he has a very mild form of the disease as death from metastatic carcinomas in early life is a common, though not inevitable characteristic. The onset of skin tumours in the present patient was not apparent until the age of 21 and was aggravated by a period spent in N. Africa. This correlates well with the necessity to use a high dose of UV irradiation to demonstrate reduced repair replication, confirming the relationship between sensitivity to sunlight and reduction in the level of unscheduled DNA synthesis (Bootsma et al 1970).

4. Christchurch Chromosome

The consistent reduction in the level of unscheduled DNA synthesis in H.W. compared with all the control used would seem to be significant. This does not appear to be associated with the fibrosis from which she was suffering as the chromosomally-normal individual with this disease (E.H.) showed no reduction. On the other hand R.B., also with a Ch' chromosome had the same level of synthesis as one of the controls and a higher level than the second (normal) control. The former was, like R.B., a cancer patient undergoing radiotherapy, and therefore probably a better control, although it is impossible to assess the significance of this difference with such limited data. The importance then, of the genetic material in the short arms of chromosome 22 is not clear from these studies. However it is quite possible that different amounts of material are missing in the two cases, H.W.

having a slightly larger deletion than R.B. however this can in no way imply that one of the loci concerned with the repair process is on chromosome 22.

5. Achiasmate Males

Of the four individuals considered, the only one showing a significantly reduced level of unscheduled synthesis compared with its control is the one with the very low chiasma count (9) and shown in Fig. V 9. In view of the complexity of a process such as meiotic crossing-over, it would be expected that many genetic loci are involved, only a proportion of which are responsible for the recombination of the DNA strands. It is therefore not surprising then, that different results were obtained for the different individuals.

This demonstration that there may be a connection between impaired meiotic crossing-over and a reduced ability to undergo unscheduled DNA synthesis following irradiation, is the first for eukaryotes and is interesting in two respects. It agrees well with results for *rec⁻* bacteria in which radiosensitivity is associated with reduced recombination (Clark and Margulies 1965; Howard-Flanders and Boyce 1966; Howard-Flanders and Theriot 1966) and also with those of Watson (1969) for c3G Drosophila. Chromosome aberration yields following X-irradiation of lymphocytes from the same individual showed a slight (20%) decrease compared with the control (Adams 1970), a similar result to that obtained for a strain of mice with low chiasma frequencies (Searle et al 1970). It had already been suggested that these two processes may have steps in common, including a certain amount of DNA repair replication (Evans 1967; Kihlman and Hartley 1968).

The model proposed by Whitehouse (1963) for crossing-over and

gene-conversion also necessitates the synthesis of short stretches of DNA, and evidence for this was first presented by Meselson (1964) for bacteriophage. Subsequently DNA synthesis during meiosis was demonstrated for liliaceous plants (Hotta et al 1966). This synthesis, occurring during prophase, represented about 0.3-0.4% of the total DNA and was temporally quite distinct from S synthesis. Its inhibition resulted in arrested development or chromosome damage (Ito et al 1967). Later work (Hotta and Stern 1971), showed that the synthesis during zygotene represents delayed replication, but that during pachytene, the stage of chiasma formation, it is of the repair type. An endonuclease, a kinase and a ligase are also active at this time (Howell and Stern 1971).

The dependence on DNA synthesis for crossing-over has also been demonstrated for Chlamydomonas (Davies and Lawrence 1967) and in E.coli integration of donor genetic determinants is sensitive to edeine, prior to the commencement of DNA replication (Wlodarczyk and Kunicki-Goldfinger 1970). Recently DNA synthesis has been demonstrated during pachytene in mouse and human cells (Kofman-Alfaro and Chandley 1971; Chandley and Kofman-Alfaro 1971). Chandley and Kofman-Alfaro have also compared the amount of unscheduled DNA synthesis following UV-irradiation at various stages during meiosis and have shown that there is a very elevated amount at this stage. They suggest that this may reflect the involvement of a recombinational repair process that is normally operative at a reduced level at this stage.

From Hotta and Stern's work it is apparent that chiasma formation and recombination is dependent on a small amount of non-semiconservative DNA synthesis and in view of the association between reduced chiasma

formation and reduced unscheduled DNA synthesis demonstrated here, it would appear that the same DNA repair mechanism may be operating in both cases. We may speculate from the evidence that this mechanism might also be involved in aberration induction.

VI GENERAL DISCUSSION AND CONCLUSIONS

Following the general acceptance that DNA, and not protein, was the repository for genetic information (see Watson, 1968), it seems to have been generally believed that the DNA in a cell was a static molecule rarely undergoing change. When such changes did occur they were assumed to be permanent and were expressed as mutations. It is only in the last decade that it has been generally realised that nuclear DNA is subject to wear and tear, and that damage can be repaired so that the DNA is restored to its original state without resulting in mutation. The experimental data presented in this thesis has been concerned with the induction of DNA synthesis following the external application of various mutagenic agents and its relevance to DNA repair mechanisms.

That DNA synthesis is immediately initiated in response to mutagenic action was clearly demonstrated in Chapter III where cells, known to be in G₁, and although able to proceed into S phase following antigenic stimulation, need 24 hours of preparative RNA and protein synthesis to do so. Such induced DNA synthesis has been described for many mammalian cell lines (see Introduction) and it may be distinguished, even in the presence of normal S synthesis, by its characteristic pattern of replication. Using a density prelabel, such as BUdR, synthesis induced by mutagens can be demonstrated as ³H-thymidine incorporation into both strands of the DNA in stretches too small to affect the density of the heavy (BUdR-labelled) strand in alkaline CsCl gradients (Rasmussen and Painter 1966). In such proliferative cell systems normal semi-conservative S synthesis only involves incorporation of ³H counts into DNA of normal density. This mutagen-induced synthesis involving both complementary strands of a DNA molecule is termed "repair replication"

(Painter and Cleaver 1968) and, using several mammalian cell lines, its extent has been correlated with that of unscheduled DNA synthesis (i.e. a synthesis of DNA outside the normal S phase) demonstrated autoradiographically or by TCA precipitation (Painter and Cleaver 1969).

For the present work a regular blood supply was available and it has been possible to study unscheduled DNA synthesis and "repair replication" following various mutagen treatments of human lymphocytes. Prior to the commencement of this project, all of the published data was on cell lines, some of which had been in culture for many years, and the response of freshly-drawn lymphocytes was considered to give a more accurate impression of the response of "normal" cells in the human body. The characterisation of UV-induced DNA synthesis as "repair replication" has been confirmed for lymphocytes and, using autoradiography, it has been possible to screen for "repair" -deficient human mutants by estimating ^3H -thymidine uptake following UV-irradiation of lymphocytes from small blood samples.

The Significance of "Repair Replication" for Cell Survival

A question of considerable importance is "what is the biological significance of the repair replication process?" Does the process result in real restoration of repair of damaged DNA thus enhancing cell survival and correcting errors that could eventually result in mutations? Studies on the repair of cellular DNA in bacteria systems have been discussed in the Introduction to this thesis. Evidence was presented for the excision of UV-induced pyrimidine dimers and for "repair replication" resulting in resynthesis of the damaged strand. In addition, mutations at loci concerned with either of these processes result in reduced survival and using such radiation-sensitive mutant

strains a large amount of information about the recovery phenomena has been obtained.

One such mutant lacking polymerase I (polAI; de Lucia and Cairns 1969) is known to be defective in excision repair, the double mutant polAI uvrA6 being only slightly more sensitive to UV than the uvrA6 single mutant (Monk et al 1971). The defect is evident in the final rejoining step in the excision repair process (Kanner and Hanawalt 1970) and "repair replication" is more extensive than normal resulting in large newly-synthesised patches (more than 1500 nucleotides - Cooper and Hanawalt in press quoted by Hanawalt 1972). The polymerase I enzyme, in the absence of known mutagenic action has also been shown to have exonuclease activity and plays an important role in removing mismatched nucleotides from the 3' hydroxyl end of a growing DNA strand (Brutlag and Kornberg 1972). The presence of this mechanism for removing mismatched bases, or the recombination repair system (described earlier) is essential for survival and is shown by the non-viability of the recA polAI double mutant (Gross et al 1971). Repair replication following thymine starvation led to the idea that transcription might introduce single-strand breaks into the DNA (Pauling and Hanawalt 1965) and the rejoining of such breaks appears to be slower in polAI mutants than in the parental strain (Nakayama and Hanawalt in press, quoted by Hanawalt 1972). In normally-growing E.coli there is a turnover of about 0.1% of parental DNA per generation and this level is reduced when RNA synthesis is inhibited (Grivell and Hanawalt in press, quoted by Hanawalt 1972).

There is a considerable amount of evidence to suggest that UV-induced damage to the DNA of mammalian cells is dealt with by a

similar "excision repair" mechanism. Post-irradiation incubation of human amnion, HeLa and WI-38 cell lines has been shown to result in a reduction in the number of thymine dimers in the DNA and their increase in the TCA-soluble fraction with increasing time post-irradiation (Regan et al 1968). However other attempts to demonstrate this in Chinese hamster (Trosko and Kasschau 1967) and mouse L-cells (Klimek 1966) have been unsuccessful.

The excision of dimers results in strand breakage and such strand breakage and rejoining can be demonstrated on alkaline sucrose gradients. Ben-Hur and Ben-Ishai (1971) have been able to demonstrate breakage and rejoining of HeLa cell DNA following exposure to UV light at 250 ergs/mm^2 . This process is completed within 8 hours and they show that rejoining is prevented by inhibitors of "repair replication" such as acriflavine (Cleaver 1969b). It is also reduced by hydroxyurea, possibly by affecting nucleotide pools; addition of all four nucleotides reverses the effect. Hydroxyurea has no effect on rejoining of X-ray induced strand breaks and it is therefore not preventing ligase activity (Sawado and Okado 1970). It is also considered to have little, if any, effect on "repair replication" (Cleaver 1969b). However the reported effect of hydroxyurea on strand rejoining (Ben-Hur and Ben-Ishai 1971) is not marked until incubation times of 90 minutes or more are studied. Since a large part of the repair replication process is complete by that time a possible effect of hydroxyurea on this process may have been undetected. It may also be significant in this connection that hydroxyurea at a similar concentration was shown by Djordjevic and Tolmach (1967) to reduce survival of irradiated (but not unirradiated) HeLa cells.

The importance of this DNA "excision repair" process for cell survival is also evident from work on cells derived from Xeroderma pigmentosum patients (Xp cells). These patients are sensitive to sunlight and are prone to the development of skin tumours. In culture their cells are also more UV-sensitive than normal showing considerably reduced survival levels (Cleaver 1970b). Xp cell lines had previously been shown to have reduced levels of "repair replication" after UV exposure (Cleaver 1968) and this reduction was correlated with the severity of the disease in a number of cases (Bootsma et al 1970). The case described in Chapter V is also consistent with this finding. Reduced levels of unscheduled DNA synthesis following UV-irradiation of Xp cells have also been demonstrated in cells in vivo, labelling of epidermal and upper dermal cell nuclei being considerable in control subjects but absent in Xp patients (Epstein et al 1970). It has also been shown that normal fibroblasts excise a large percentage of induced pyrimidine dimers within a 24-hour post-irradiation incubation period and that this ability is lacking in fibroblasts from Xp patients (Cleaver and Frosko 1970). There is therefore a correlation, for cells in vitro derived from Xp patients, between survival level, excision of pyrimidine dimers and "repair replication". This is paralleled in vivo by increased sensitivity to sunlight, resulting in a high incidence of skin tumours, also reduced levels of unscheduled DNA synthesis, and it is tempting to conclude that there is a correlation between an inability to repair environmentally-induced DNA damage and malignant transformation. However "repair" is evident in another genetically determined syndrome (Rothmund-Thomson) showing a high incidence of skin cancer, and also in malignant cells such as HeLa

(Cleaver 1970c).

It was likewise impossible in the present work to implicate the process of "excision repair" in the abnormalities associated with Bloom's syndrome and Fanconi's anaemia. Both of these syndromes are associated with increased incidence of leukaemia and spontaneous chromosome rearrangements. The occurrence of spontaneous unscheduled DNA synthesis (Djordjevic et al 1969) suggests that a certain amount of "repair" is important for maintaining the structural integrity of the genetic material, possibly in a manner similar to that just described for bacteria. Hydroxyurea-resistant synthesis in the absence of mutagens has also been demonstrated in the present work in both nonstimulated and stimulated cells (in the latter, about 4% of the total DNA synthesised, see fig. IV 3) and this may represent a similar phenomenon. An association between a declining capacity to carry out some form of "DNA maintenance", leading to the accumulation of somatic mutations has been postulated as a mechanism of ageing (Szilard 1959). It is interesting that the results for the Fanconi family described in Chapter V show lower levels of unscheduled DNA synthesis in the adults as compared with the children. However cells from patients with "progeria" a syndrome associated with premature ageing, are able to carry out "repair replication" (Cleaver 1970c).

It has also been impossible to correlate chromosome rejoining following X-irradiation with levels of unscheduled DNA synthesis. Protein synthesis inhibitors and not DNA synthesis inhibitors are effective in preventing rejoining of such breaks (Wolff 1960) and rejoining of chromosome breaks in the absence of unscheduled DNA synthesis has been shown for rat kangaroo cells (Shaeffer et al 1971).

Wolff and Scott (1969) were unable to correlate the 2 processes using mammalian cells with differing levels of "repair replication".

However Parrington et al (1971) have shown an increased incidence of chromosome aberrations in UV-irradiated Xp fibroblasts.

That "excision repair" is not restricted to correcting UV-induced damage is indicated by the many demonstrations of unscheduled DNA synthesis and "repair replication" following X-irradiation and chemical mutagens (see Introduction). Excision of alkylating products has also been demonstrated (Papirmeister and Davison 1964) and Roberts et al (1971b) showed that the kinetics of "repair replication" resembled those of the loss of alkyl groups following alkylation of hamster cells DNA with MNNG and that approximately 100 nucleotides were built into the DNA for every methyl group eliminated. However the rate of excision of alkylated moieties and the ability to carry out "repair replication" following mustard gas treatment could not account for a 20-fold difference in drug sensitivity in 2 lines of Yoshida sarcoma cells (Ball and Roberts 1970).

Additional support for the idea ~~that~~ "repair replication" is part of a process that enhances cell survival comes from a study of the subsequent replication of DNA following irradiation of cells. In diploid W1-38 cells Painter et al (1970) have shown that DNA that has undergone "repair replication" following UV irradiation of the cells can later participate in semi-conservative replication and that the fraction that does so is inversely proportional to the UV dose level. This suggests that despite the fact that such synthesis is evident at very high mutagen dose levels, where survival is low, it is probably able to cope with small amounts of damage, in particular

those incurred during normal cellular functions, and this could account for the spontaneous unscheduled DNA synthesis observed in the absence of mutagen treatment described by Djordjevic et al (1969).

The Mechanisms of "Repair" as Revealed by Mutant Genotypes

In the present work the unscheduled DNA synthesis detected in TCA-insoluble cell extracts as a consequence of UV irradiation and detected autoradiographically in the experiments described in Chapter V, has been shown to have the same temporal pattern as "repair replication" following UV irradiation. The same holds for HN2-induced synthesis and both unscheduled DNA synthesis and "repair replication" are assumed to be the same process lasting some 4 to 6 hours following treatment. In contrast, γ -ray and MMS-induced unscheduled DNA synthesis are more extended, lasting the full 22 hours incubation time used in the experiments. It was concluded that this difference reflected differences in the type of damage induced by these agents, the former producing distortions of the DNA backbone, and the latter strand breakage. A similar pattern of cell killing in response to these agents is evident in microorganisms. Strains of E.coli differing in their response to cell killing by X-rays (e.g. B/r versus B_{s-1}) show a similar response to the lethal effects of UV light and HN2 (Haynes et al 1964). Moreover, B_{s-1} strains that are sensitive to X-rays are also sensitive to MMS, whereas hcr mutants may be resistant to both X-rays and MMS but sensitive to UV and HN2 (Bridges and Munson 1966). Similar parallellisms are to be observed with Saccharomyces where UV sensitivity often correlates with HN2 sensitivity and X-ray and MMS sensitivity are associated (Brendel et al 1970). However, it should be emphasised that in both organisms mutants have been isolated which

are sensitive to all four mutagens, thus indicating that at least part of a repair mechanism may be common to all forms of damage produced by these mutagens.

Xp cells are also interesting in this connection in emphasising the importance of the type of damage in the "repair replication" process. As described earlier, Xp cells are abnormally sensitive to UV irradiation and show reduced "repair replication". However normal amounts of synthesis are evident after X-irradiation, or after UV irradiation of Xp cells in which BU is incorporated into the DNA (Cleaver 1969c). Strand rejoining following X-irradiation is also normal (Kleijer et al 1970). It is assumed from these results that it is the initial endonuclease step in the excision repair process that is lacking in Xp cells and Setlow et al (1969) have shown that the expected reduction in molecular weight of DNA associated with dimer excision is not evident. Cleaver (1971) has also shown that normal amounts of synthesis follow MMS and MNNG treatment of Xp cells and this is consistent with the idea that these mutagens result in DNA strand breakage. In Yoshida sarcoma cells, increased resistance to HN2 is associated with increased resistance to UV irradiation; such resistant cell lines show similar sensitivities to MMS and X-rays (Fox and Fox 1971/72).

The results in Chapter IV for "repair replication" are not so easily explained. The UV- and HN2-induced synthesis show a similar temporal pattern to that for unscheduled DNA synthesis and it is likely that it is due to resynthesis following excision of the damaged bases. Following γ -irradiation an initial fast period of synthesis, with the characteristics of "repair replication", shares the same kinetics as strand rejoining (Donlon and Norman 1971) and it is tempting to

conclude that these two processes are associated. It is likely that a similar process follows MMS treatment, although it appears to be somewhat more prolonged, probably due to the fact that strand breakage is a secondary reaction resulting from hydrolysis of the methylated bases (Strauss and Hill 1970). The more extended synthesis following these two mutagens is more difficult to account for. Excision of alkylation products lasts at least 9 hours following MNNG treatment of hamster cells (Roberts et al 1971b) and at least 24 hours following mustard gas treatment of Yoshida sarcoma cells (Ball and Roberts 1970). It is therefore likely that the extended synthesis following MMS is a form of "repair replication". It is evident that at least some of the newly-synthesised regions are relatively long, although these are associated with the initial sites of "repair".

In contrast, the synthesis subsequent to the "repair replication" of the first 2 hours post- γ -irradiation, is in the form of longer molecules which, when labelled with BUdR, band separately in CsCl gradients. The many unsuccessful attempts to demonstrate degradation following X-irradiation of mammalian cells (Little 1968; Painter 1968; Hill 1969) also suggest that this is not a form of "repair replication". The exonuclease experiments show that some of the incorporated ^3H -thymidine could be in the form of end-addition, however it is not possible to say whether it is enough to account for this synthesis (approximately half of the total at 50 k rads) especially as similar results were obtained for the MMS-induced incorporation

Undoubtably the processes of "DNA repair" are of great importance, being present in all types of cells and all kinds of organisms. In man, unscheduled DNA synthesis has been demonstrated in many tissues

including very specialised cells such as skeletal muscle (Stockdale 1971) and polymorphonuclear leucocytes (see Fig. V 1) which have no proliferative ability. On the other hand some of the most primitive organisms such as the mycoplasma, whose genome codes for only some 700 proteins, have been shown to have the necessary enzymes for "repair replication".

Evidence is accumulating, at least for bacteria, that the primary function of "repair replication" is in relation to normal cellular processes such as transcription and genetic recombination, and that its association with DNA damage following exposure to external mutagens may be of secondary importance. In mammalian cells, unscheduled DNA synthesis is much more easily detected following mutagen exposure and it is assumed that this represents a similar process of "repair replication" to that occurring normally in the cell. At low doses of mutagen where survival is high, this is probably true. At higher doses, unscheduled DNA synthesis may also reflect the same "repair" phenomenon despite the fact that most of the cells are destined to die. However, high X-ray doses result in extensive DNA synthesis that, in the experiments described here, does not appear to be "repair replication" and it may be that some of this unscheduled DNA synthesis represents some other process, not associated with "repair".

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APPENDIX

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The parallelism observed in unscheduled DNA synthesis in human cells exposed to UV light and HN₂, and human cells exposed to γ -rays and MMS, recalls similar parallel responses to cell killing by these agents in certain microorganisms. Strains of *E. coli* differing in their response to cell killing by X-rays (*e.g.* B/t *versus* B_{s-1}) show a similar response to the lethal effects of UV light and HN₂²⁵. Moreover, B_{s-1} strains that are sensitive to X-rays are also sensitive to MMS, whereas *hcr* mutants⁶ may be resistant to both X-rays and MMS but sensitive to UV and HN₂. Similar parallelisms are to be observed with *Saccharomyces* where UV sensitivity often correlates with HN₂ sensitivity and X-ray and MMS sensitivity are associated⁴. However, it should be emphasised that in both organisms mutants have been isolated which are sensitive to all four mutagens, thus indicating that at least part of a repair process may be common to all forms of damage produced by these mutagens. It is pertinent to note that CLEAVER¹¹ has recently shown that human fibroblasts (from Xeroderma pigmentosum patients) which are unable to undergo repair replication after exposure to UV light, but which show a normal response after X-radiation⁸, show a normal amount of repair replication after exposure to MMS and also MNNG.

In conclusion, our results on the patterns of unscheduled DNA synthesis induced in human lymphocytes indicate that although different rate limiting steps are involved with the four mutagens studied, common mechanisms are implicated on the one hand in the case of UV and HN₂ damage, and on the other with MMS and γ -ray-induced damage. In the former case it would appear that excision repair occurs in response to structural alterations in the DNA backbone, whereas at least part of the synthesis observed after γ -ray and MMS treatment may be associated with the repair of mutagen-induced strand breaks.

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tween MMS and γ -rays. However, strand breakage following MMS treatment is a secondary reaction resulting from hydrolysis of the alkylated purines to give apurinic sites, and this would account for a less obvious initial fast reaction comparable to that obtained with X- or γ -rays.

In contrast to the results obtained with MMS and γ -rays, the bulk of induced DNA synthesis following UV light or HN₂ treatment is largely over within 3 h and is complete after 6 h. This is true over a very wide range of doses for both mutagens. We have found (unpublished work) that the label that is incorporated into DNA during the 6 h following exposure of lymphocytes to UV light, is incorporated into both strands of the DNA and that this "repair synthesis" shows the same temporal patterns as unscheduled synthesis. Similar experiments by ROBERTS *et al.*⁴⁸ on repair synthesis in HeLa cells exposed to sulphur mustard, show a somewhat extended, but very similar temporal pattern, with one half of the induced synthesis in the BUdR-substituted strand being completed within 3 h following mutagen treatment.

The different types of lesions induced in DNA by γ -rays and MMS on the one hand and UV light and HN₂ on the other may account for the different patterns of unscheduled DNA synthesis that are observed between these mutagens. Among the main and most important products in DNA exposed to UV-irradiation are the *intra-strand*, pyrimidine dimers, and in particular the thymine dimer. It has been shown from bacterial studies that the thymidine dimer interrupts normal DNA replication and that such replication is resumed when the dimers are excised; moreover, the excision of such dimers is accompanied by an increase in cell survival⁵⁰. Thymine dimer excision has also been demonstrated to occur in mammalian cells after exposure to UV (see INTRODUCTION) and it is presumed that such excision is an early step in the repair process that results in the incorporation of labelled precursor into damaged DNA.

In the case of bifunctional alkylating agents, it is usually considered that the important lesion in DNA is the cross linking, *via* alkylation, of two guanines on opposite strands of the DNA^{7,23,35}. Both monoguanyl and diguanyl alkylation products are excised from the DNA of bacterial^{32,39} and mammalian cells^{14,46} exposed to bifunctional alkylating agents; but there is some disagreement as to the relative preference for the elimination of diguanyl over monoguanyl products. Studies on HeLa cells¹⁴ suggest that there is a random excision of alkylated products along both strands of the DNA, whereas in mouse L-cells, REID AND WALKER⁴⁶ find that the loss of renaturable, *i.e.* cross-linked DNA, has a half life of 2 h compared with 18 h for the loss of total alkylated products, *i.e.* a preferential loss of diguanyl products. In *E. coli* B/r, LAWLEY AND BROOKES³² and VENITT⁵⁶ report that some 50% of isotopically labelled HN₂, and including all diguanyl products, are excised within 80 min following treatment.

Excised diguanyl alkylation products are often believed to be largely derived from *interstrand* cross links. Recent evidence, however^{20,33}, has indicated that *intra-strand* cross links between adjacent guanines on a given DNA strand may be three or four times more frequent than *interstrand* cross links. Such *intrastrand* diguanyl products formed following HN₂ treatment are formally comparable to the *intrastrand* pyrimidine dimers produced by UV. Both products are *intrastrand* alterations that will produce conformational changes within the DNA helix, and both might be expected to be subjected to the same mechanism of excision repair.

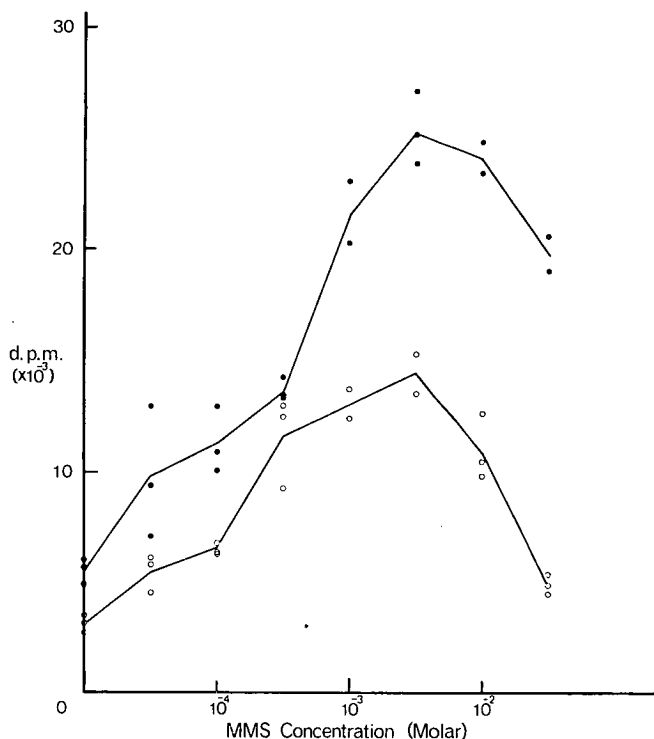


Fig. 13. $[^3\text{H}]$ Thymidine uptake following a 1-h incubation in various doses of MMS. ○, shows uptake during the first 3 h after treatment, 3 samples for each dose. Three further samples were incubated in "cold" medium for 3 h and ●, shows uptake during the subsequent 3 h. (cf. Fig. 10)

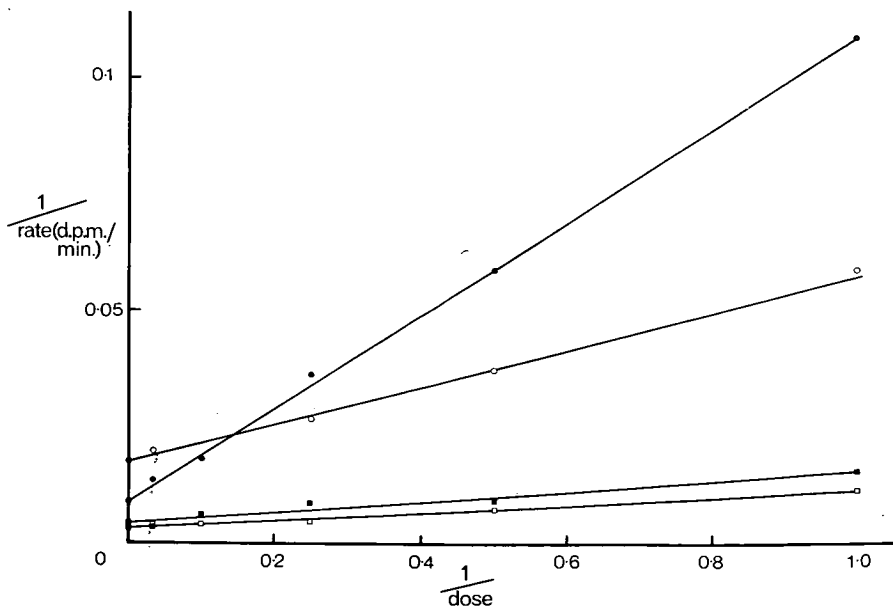


Fig. 14. Michaelis-Menten plot of initial rates of incorporation of $[^3\text{H}]$ thymidine after each mutagen. Reciprocals of the initial rates are plotted against the reciprocal of dose. For each mutagen five doses in the ratios of 1:2:4:10:30 were used. ●, HN₂; ○, MMS; ■, γ-rays; □, UV light.

after exposure to ionizing radiations might be involved in the rejoining of single strand breaks in the DNA, a rejoining process which molecular weight studies have shown occurs largely during the first 60 min post-irradiation^{28,29,34,36}. This interpretation is strengthened by the recent work by DONLON AND NORMAN¹⁶ who have compared the kinetics of rejoining of single-strand breaks in the DNA of X-irradiated lymphocytes with the kinetics of unscheduled DNA synthesis in these cells. Their results indicate that the repair of single-strand breaks and the stimulated incorporation of nucleotides share the same rate-limiting enzymatic step.

BRENT AND WHEATLEY⁵ have shown that in HeLa cells exposed to X-ray doses of around 5 krad, "repair-synthesis", as measured by incorporation of BUdR into normal density DNA, is completed during the first two hours post-irradiation. It is therefore possible that the slower process of incorporation of label into DNA that we observe after the first 60 min following X-irradiation, and continuing for at least 22 h, does not reflect a repair-replication process. However, this process is of increasing importance at higher doses and our data show that it may account for the majority of label incorporated into the DNA. It is possible that the slow process may be associated with damage other than single-strand breaks. Alternatively breaks may develop some considerable time after the absorption of the radiation energy, or, as suggested by FOX AND FOX²¹ for the late synthesis in lymphoma cells exposed to MMS, may represent a random addition of label onto broken ends of the DNA.

Exposure to MMS is known to result in DNA strand breakage^{54,55} and it may be therefore not surprising that the pattern of induced DNA synthesis is similar be-

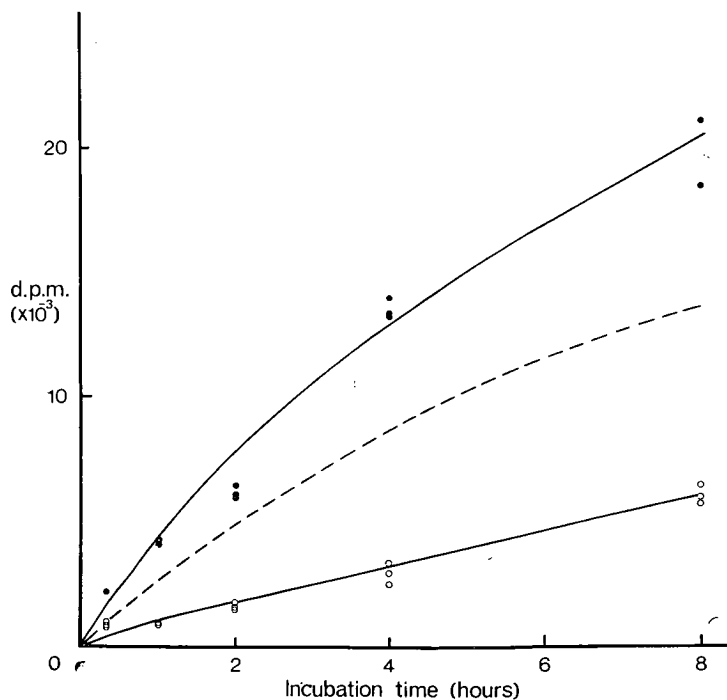


Fig. 12. [³H]Thymidine uptake following a 1-h incubation at 37° in 10⁻³ M MMS, and continuous incubation in "hot" medium. The results from three cultures at each point and the net incorporation are shown as in Fig. 5. ○, Control; ●, treated.

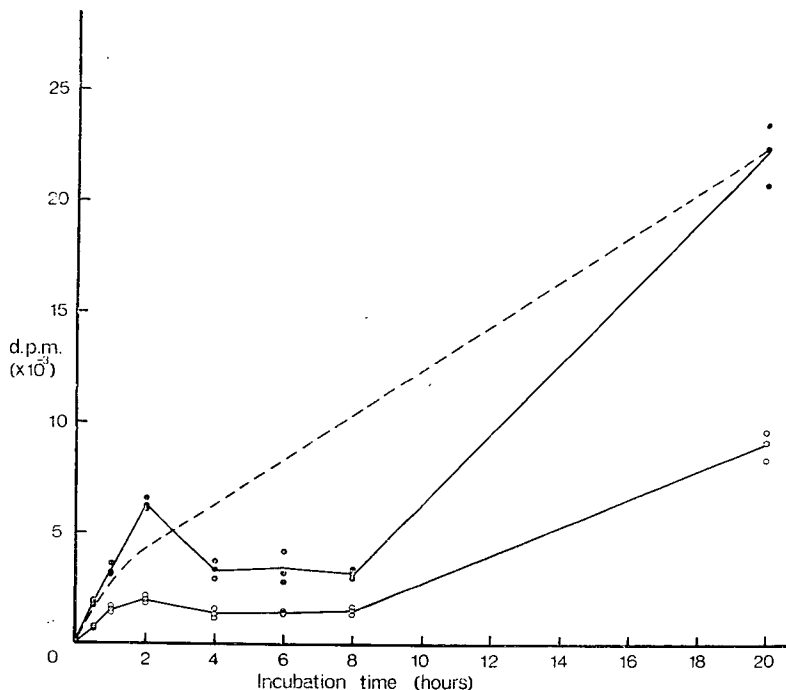


Fig. 11. $[^3\text{H}]$ Thymidine uptake following a 10-min incubation in $10^{-3} M$ MMS. Three samples were analysed at each time and the d.p.m. values represent uptake of $[^3\text{H}]$ thymidine over the 2 h prior to sampling, the cells first being incubated in "cold" medium. The incorporation at 20 h represents uptake from 8–20 h. Net counts are shown as before and should be compared with Fig. 6. $\circ$, Control; $\bullet$, treated.

Since $1/V$ plotted against $1/S$ gives a straight line, the reaction is shown to be dependent on the free enzyme concentration (E). The fact that with the present data the rate at infinite dose (damage) differs for the different mutagens (except as between UV light and γ -rays) might therefore be taken to imply that different enzymes are involved in the rate-limiting steps for the damage induced by these mutagens.

DISCUSSION

The most striking feature of these results is the way in which the patterns of thymidine incorporation after exposure of G_1 human lymphocytes to four types of mutagen fall into two categories. Synthesis of DNA following exposure to ionising radiation or to MMS treatment continues for at least 22 h after exposure, whereas after exposure to UV or HN₂, induced synthesis is completed within 6 h.

The γ -ray results are in accord with and extend previous results for induced unscheduled DNA synthesis following X-irradiation of human lymphocytes⁵² and mouse L-cells²⁶. SPIEGLER AND NORMAN⁵³ have stressed the likely involvement of two reactions with different rates, a fast reaction which is completed in the first hour following exposure and a slow reaction that continues for at least 7 h. It was suggested that the initial fast reaction might involve the incorporation of only one or two nucleotides per lesion. It is tempting to conclude that the fast reaction process observed

TABLE I
INTERCEPTS OF EXTRAPOLATED MICHAELIS-MENTEN PLOTS FOR UV LIGHT, γ -RAYS, HN₂ AND MMS

Mutagen	Intercept	S.E.
UV	3.18	0.357
γ -Rays	4.28	1.209
HN ₂	8.88	1.045
MMS	17.43	1.195

and $6 \cdot 10^{-3}M$. [³H]Thymidine uptake after 0, 7, 15, 22 and 30 min post-treatment was measured for each mutagen and the rates of incorporation were determined from the slopes of the lines of least squares that was fitted to each set of data. The reciprocal of the rate of incorporation for each dose was then plotted against the reciprocal of dose for each mutagen (Fig. 14) and in each case a straight line graph was obtained. The intercept of each extrapolated line onto the Y axis gives a measure of the rate on that line where the dose is infinitely large; these intercepts are shown in Table I.

In the Michaelis-Menten equation

$$V = \frac{E}{1 + K/S}$$

where E = free enzyme concentration; S = concentration of radiation induced substrate (lesions); V = rate; and K = rate constant. This equation can be rearranged to give

$$\frac{E}{V} = 1 + \frac{K}{S}$$

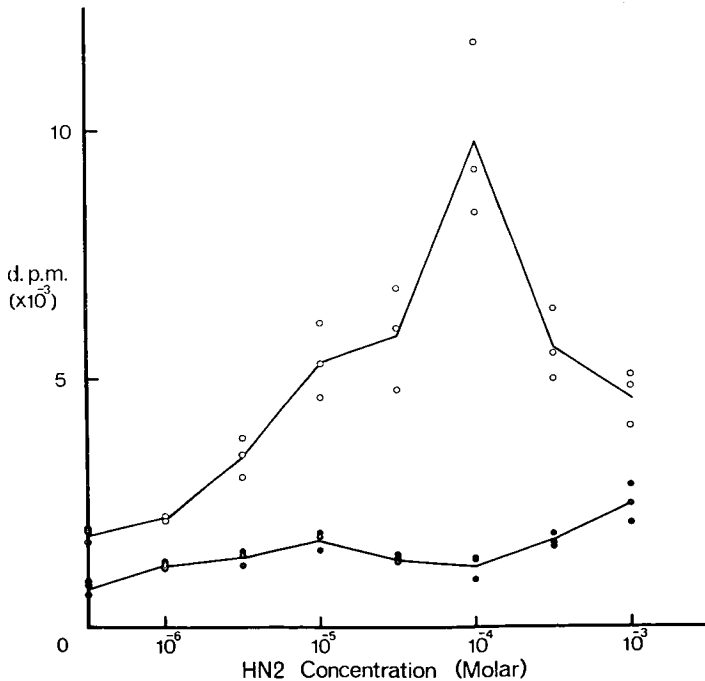


Fig. 10. [³H]Thymidine uptake following a 1-h incubation in various doses of HN₂. O, shows uptake during the first 3 h after treatment, 3 samples for each dose. Three further samples were incubated in "cold" medium for 3 h and ●, shows uptake during the subsequent 3 h.

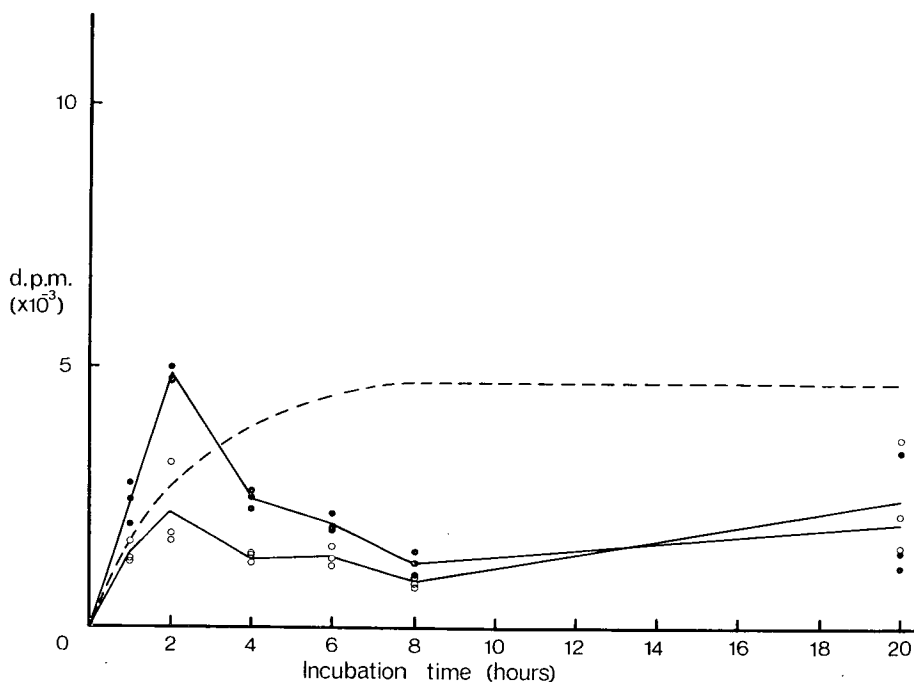


Fig. 9. $[^3\text{H}]$ Thymidine uptake following a 1-h incubation in $10^{-5} M$ HN2. Three samples were analysed at each time and the d.p.m. values represent uptake of $[^3\text{H}]$ thymidine over the 2 h prior to sampling, the cells first being incubated in "cold" medium. The incorporation at 20 h represents uptake from 8–20 h. Net counts are shown as before and should be compared with Fig. 2. $\circ$, Control; $\bullet$, treated.

(4) MMS

Experiments similar to those with HN2 were carried out. In Fig. 11 the uptake of $[^3\text{H}]$ thymidine into the DNA of cells treated with $10^{-3} M$ MMS for 10 min and pulse labelled is shown. Uptake continues for at least 21 h after exposure in a manner similar to that observed after γ -ray irradiation. However, the initial rapid rate characteristic of the γ -ray data is not as marked, although there is an indication of a decrease in rate at 2 h. A similar result was obtained at a dose level of $3 \cdot 10^{-3} M$ and also with a 1-h incubation in $10^{-3} M$ MMS followed by continuous exposure to $[^3\text{H}]$ thymidine labelling (Fig. 12).

The incorporation at different dose levels was measured in the same way as in the HN2 experiments and was found to increase with increasing dose up to around $3 \cdot 10^{-3} M$ and then decline at higher concentrations (Fig. 13). In contrast with HN2 there is a considerable amount of $[^3\text{H}]$ thymidine taken up between 3 and 6 h after exposure, in fact as much as that during the first 3 h (*cf.* Figs. 10 and 13).

(5) Initial rates of $[^3\text{H}]$ thymidine incorporation for all mutagens

The rates of $[^3\text{H}]$ thymidine uptake over the first 30 min post-treatment for a variety of doses of each mutagen were compared. The following doses were used: UV light: 0, 5, 10, 20, 50 and 150 erg/mm^2 . γ -Rays: 0, 1, 2, 4, 10 and 30 krad. HN2: 0, 10^{-5} , $2 \cdot 10^{-5}$, $4 \cdot 10^{-5}$, $1 \cdot 10^{-4}$ and $3 \cdot 10^{-4} M$. MMS: 0, $2 \cdot 10^{-4}$, $4 \cdot 10^{-4}$, $8 \cdot 10^{-4}$, $2 \cdot 10^{-3}$

(3) *HN2*

[^3H]Thymidine incorporation following various incubation periods after a 1-h treatment at 37° with $10^{-4}M$ and $10^{-5}M$ *HN2* are shown in Figs. 8 and 9. The pattern of incorporation very closely resembles that following UV-irradiation in that synthesis had been nearly completed by 4 h. In the first experiment $10^{-4}M$ *HN2* was followed by continuous [^3H]thymidine for up to 8 h resulting in 3 times the incorporation found in the controls. After $10^{-5}M$ *HN2* and pulse labelling (Fig. 9), a similar pattern of uptake occurs, although somewhat reduced in total amount. As with the UV light experiments there is no further incorporation of label between 6 and 20 h following exposure (*cf.* Figs. 8 and 9 and Figs. 1 and 2).

The incorporation of label into the DNA of cells following exposure to different concentrations of *HN2*, and post-incubation for 3 or 6 h [^3H]thymidine, is shown in Fig. 10. Uptake increases with increasing dose between $10^{-6}M$ and $10^{-4}M$ and then falls at higher concentrations. Similar falls at high dose levels are observed with UV and MMS (*cf.* Figs. 3, 13 and see below). It is evident that the incorporation at all concentrations between the third and sixth hour of incubation following *HN2* treatment is small compared with that over the first 3 h, confirming that the rate of synthesis is independent of dose over the range studied, an independence similar to that found after UV.

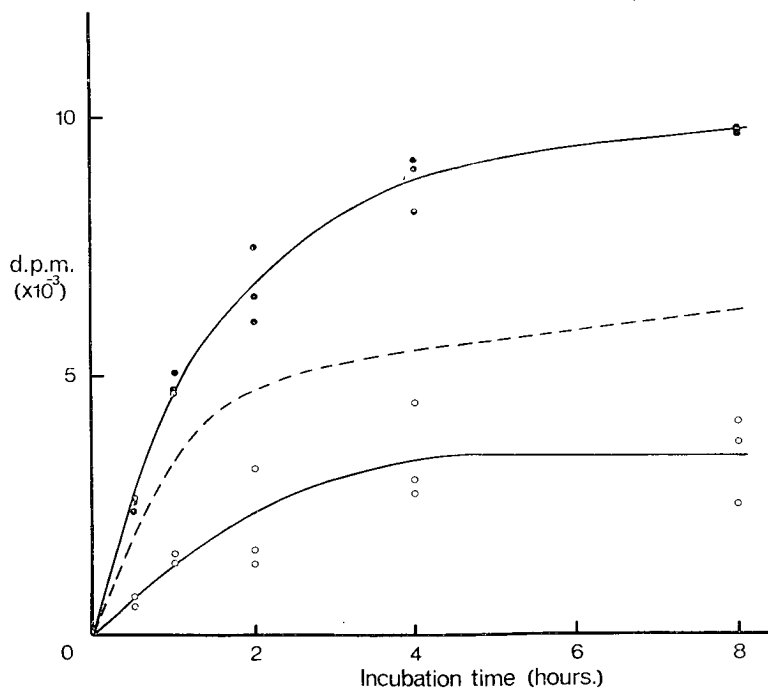


Fig. 8. [^3H]Thymidine uptake following a one hour incubation at 37° in $10^{-4}M$ *HN2*, and continuous incubation in "hot" medium. The results from three cultures at each point and the net incorporation are shown as in Fig. 1. ○, Control; ●, treated.

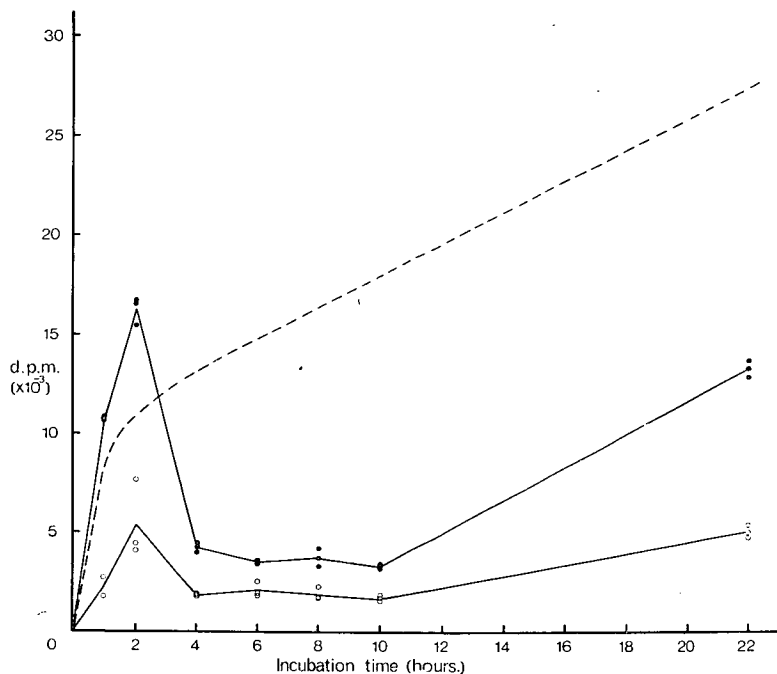


Fig. 6. $[^3\text{H}]$ Thymidine uptake after 5000 rad of γ -rays. Three samples (cultures) were analysed at each time and the d.p.m. values represent uptake of $[^3\text{H}]$ thymidine over the 2 h prior to sampling, the cells first being incubated in "cold" medium. The incorporation at 22 h represents uptake from 10–22 h. The dotted line shows the integrated net counts. $\circ$, Control; $\bullet$, treated.

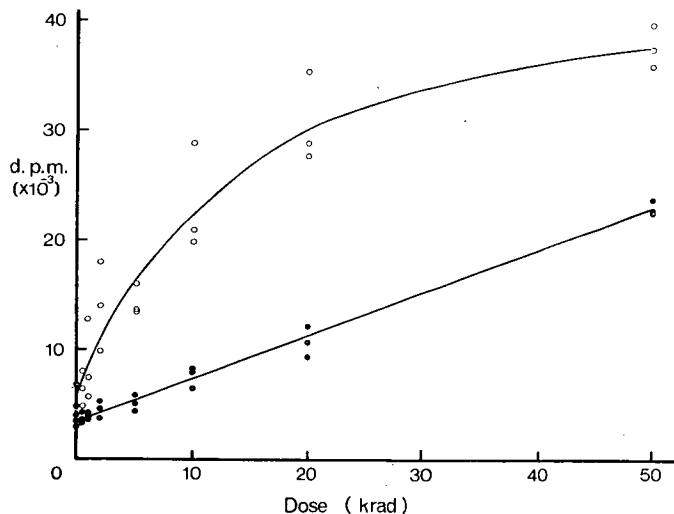


Fig. 7. $[^3\text{H}]$ Thymidine uptake after exposure to various dose levels of ^{60}Co γ -rays at 1 krad/min. $\circ$, shows uptake by cells incubated at 37° during the first hour post-irradiation. $\bullet$, cells incubated in "cold" thymidine 0–2 h and "hot" thymidine 2–4 h post-irradiation.

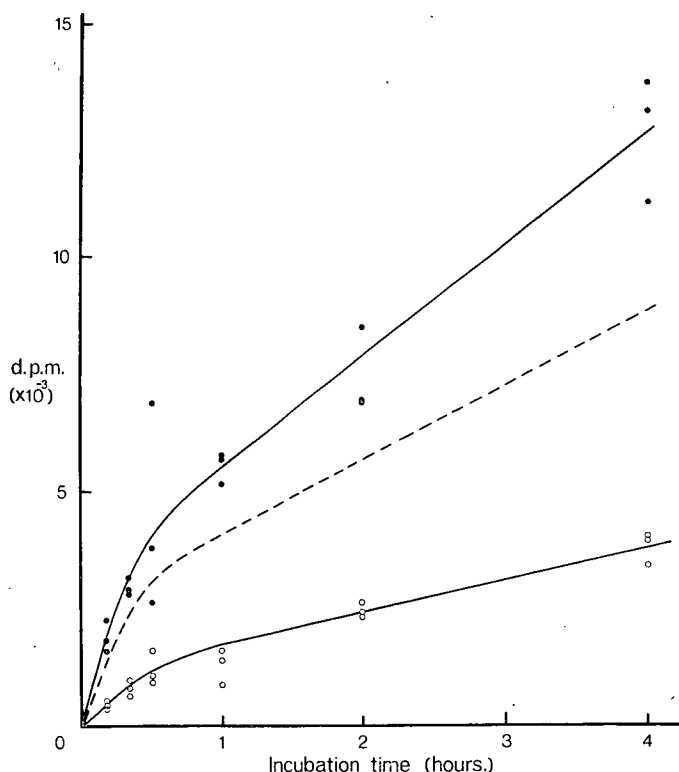


Fig. 5. $[^3\text{H}]$ Thymidine uptake following 5000 rad of γ -rays and continuous incubation in "hot" medium. Results from three separate cultures are shown for each time, and the net incorporation is shown as a dotted line. $\circ$, Control; $\bullet$, treated.

mined. In the first $\frac{1}{2}$ –1 h after irradiation there is a rapid, apparently linear, increase in incorporation of label and this is followed by a continued linear increase at a quarter of the maximum rate thereafter. Unlike the results obtained with UV light, the incorporation continues up to at least 22 h after exposure, with a constant rate from 2 h onwards.

The uptake of label after a wide range of γ -ray doses at room temperature followed by a constant incubation time at 37° , was also studied (Fig. 7). A dose rate of 1 krad/min and dose levels of 0, $\frac{1}{2}$, 1, 2, 5, 10, 20 and 50 krad were used. Following exposure, one half of the cells were incubated in $[^3\text{H}]$ thymidine for 1 h in an attempt to estimate the dose response for the initial fast reaction. However, at the low dose rates available the data obtained from high levels of exposure are not particularly informative. For the doses considered, increasing exposure increases the incorporation. This effect is most marked at low doses and the pattern is similar to the exponential increase observed following various UV doses. When $[^3\text{H}]$ thymidine is present 2–4 h following irradiation a linear increase in incorporation with dose is observed. The reaction is therefore directly dependent on the amount of damage induced and may well therefore be controlled by a different enzyme system.

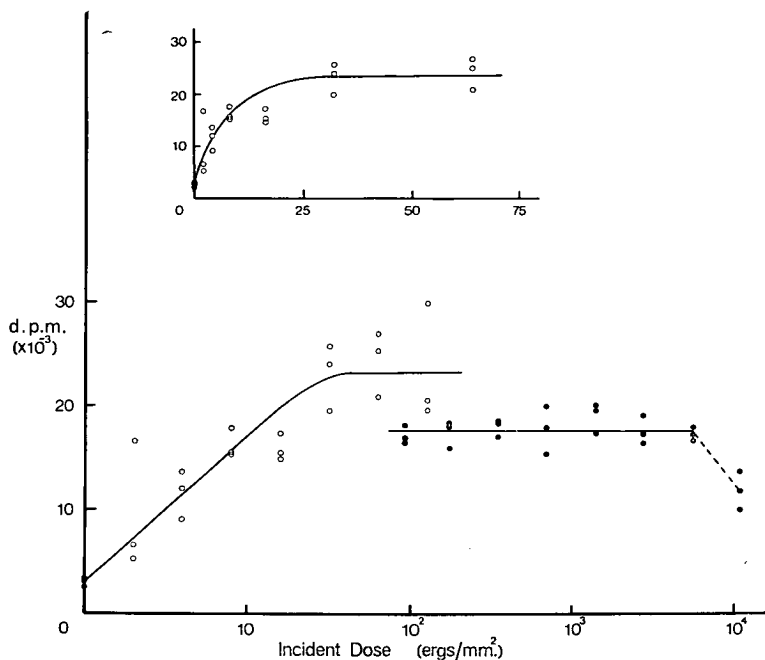


Fig. 3. $[^3\text{H}]$ Thymidine uptake during a 3-h incubation following various doses of UV light. $\circ$, shows doses up to 128 erg/mm² given at an incident dose of 1 erg/mm²/sec. $\bullet$, shows results from a second experiment involving higher doses given at an incident dose of 23 erg/mm²/sec. Inset shows low doses plotted on a linear scale.

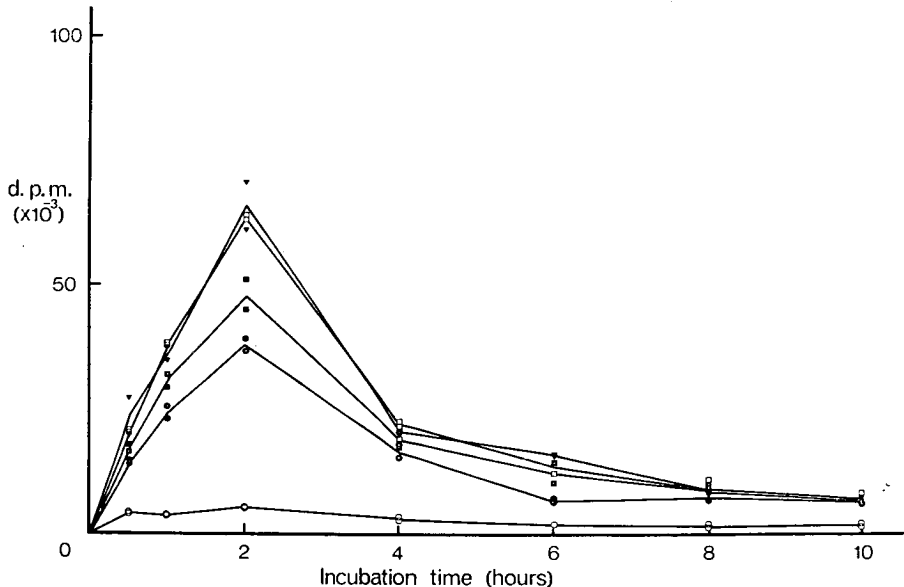


Fig. 4. $[^3\text{H}]$ Thymidine uptake after exposure to various doses of UV light up to 100 erg/mm² given at a dose rate of 1 erg/mm²/sec. Two samples were analysed at each time and the d.p.m. values represent uptake of $[^3\text{H}]$ thymidine over the 2 h prior to sampling, the cells first being incubated in "cold" medium. $\circ$, Control (no UV); $\bullet$, 10 sec exposure; $\blacksquare$, 20 sec exposure; $\square$, 50 sec exposure; $\blacktriangledown$, 100 sec exposure.

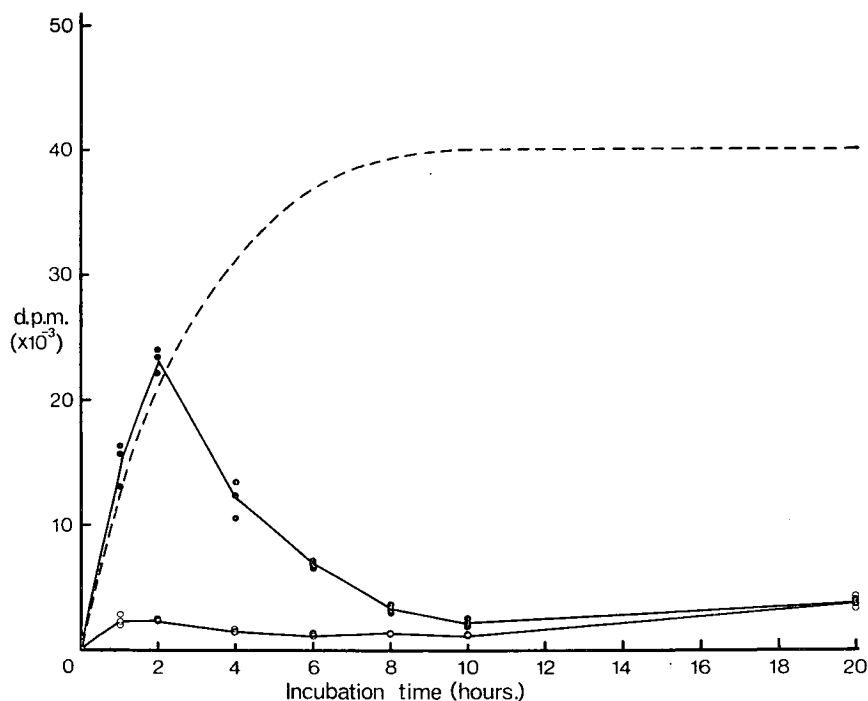


Fig. 2. $[^3\text{H}]$ Thymidine uptake after 15 sec exposure to UV light at $23 \text{ erg/mm}^2/\text{sec}$ (350 erg/mm^2). Three samples (cultures) were analysed at each time and the d.p.m. values represent uptake of $[^3\text{H}]$ thymidine over the 2 h prior to sampling, the cells first being incubated in "cold" medium. The incorporation at 20 h represents uptake from 10–20 h. The dotted line shows the integrated net counts. O, Control; ●, treated.

thymidine incorporated is reduced to about one half of the maximum value. A striking feature of all the dose response experiments with UV light is the very high sensitivity of the system. Considerable $[^3\text{H}]$ thymidine incorporation is observed at very low exposure levels, with 50% of the maximum possible incorporation being obtained at a dose of 4 erg/mm^2 (see insert in Fig. 3) and saturation occurring at around 30 to 40 erg.

The time curves of incorporation shown in Figs. 1 and 2 were obtained at doses at which synthesis is maximal. In Fig. 4 uptake (determined by 2-h "pulse labelling") for the first 10 h post-irradiation is shown for 10, 20, 50 and 100 sec exposures at incident dose rate of $1 \text{ erg/mm}^2/\text{sec}$. The same temporal pattern is obtained at each dose level, the rate being proportional to the amount of damage up to 50 sec exposure. In each case some 75% of the synthesis is complete in 4 h and the rate of incorporation falls off very markedly at this time.

(2) γ -Rays

The results for UV light relating the level of incorporation to time after exposure and shown in Figs. 1 and 2, should be contrasted with those for comparable experiments with γ -rays (Figs. 5 and 6). In the ^{60}Co γ -ray experiments in each case cells were exposed to 5 krad and the pattern of incorporation over 4 h in the continuous presence of $[^3\text{H}]$ thymidine (Fig. 5) and over 20 h after pulse labelling (Fig. 6) deter-

RESULTS

(1) *UV-light*

[^3H]Thymidine incorporation over periods of up to 20 h post-irradiation is shown in Figs. 1 and 2. Fig. 1 shows a typical result obtained when the cells were given a 1-min UV-light exposure at an incident dose of $23 \text{ erg/mm}^2/\text{sec}$ and incubated in ^3H -medium ("continuous labelling") for up to 8 h. Synthesis is seen to be essentially completed within the first 4 h following exposure and the amount of label incorporated is some 3 to 4 times that in the unirradiated controls. After 15 sec exposure and 2 h "pulse labelling" at different times following irradiation, a similar result is obtained (Fig. 2), the rate of incorporation declining over the first 6 h, at which time synthesis is virtually complete.

Fig. 3 shows the amount of [^3H]thymidine incorporated in the first 3 h following exposures at various dose levels of UV-irradiation in two separate experiments. At low doses the UV source was placed at a greater distance from the cells to give an incident dose rate of $1 \text{ erg/mm}^2/\text{sec}$. Uptake of label increases with increasing dose up to exposures of about 40 sec and then becomes dose independent up to 5600 erg/mm^2 . After 8 min at $23 \text{ erg/mm}^2/\text{sec}$ (incident dose of 11000 erg/mm^2) the amount of [^3H]-

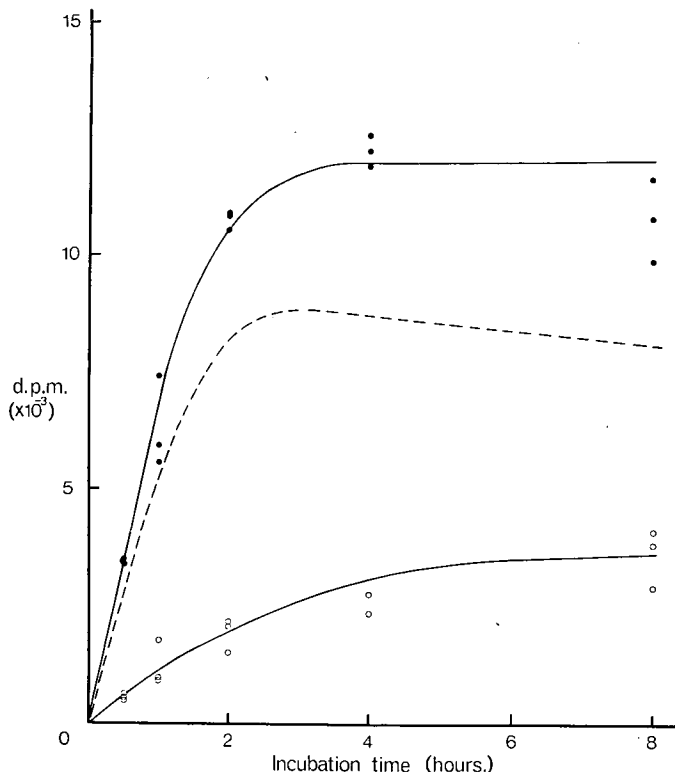


Fig. 1. [^3H]Thymidine uptake following a one-min exposure to UV light at an incident dose of $23 \text{ erg/mm}^2/\text{sec}$ (1400 erg/mm^2) and continuous incubation in "hot" medium. Results from three separate cultures are shown for each time, and the net incorporation is shown as a dotted line. O, Control; ●, treated.

cells/ml) were transferred to 10-cm glass petri dishes. The cell suspension formed a layer 3–4 mm deep and in order to obtain a uniform exposure this was continuously stirred with a magnetic stirrer during irradiation. The cells were irradiated at room temperature ($\sim 20^\circ$) from above with UV light at 2537 Å ("Hanovia" low-pressure mercury vapour lamp), the incident dose at the surface of the suspension being 23 erg/mm²/sec or 1 erg/mm²/sec. After irradiation, cells given the same exposure were pooled. Control cells were handled in the same way except that they were not exposed to UV-radiation. The UV lamp was calibrated using a biological and a physical method. The biological method involved the construction of a survival curve for *E. coli* B/r²⁵. A Latarjet dosimeter³¹ was also used, giving the same result as the survival curve.

(b) *γ -Rays*. The leucocytes were suspended in Eagle's medium instead of saline at a concentration of $1-2 \cdot 10^7$ cells/ml. Ten to twenty ml of suspension were introduced into each of two 25-ml glass universal bottles; one batch was irradiated at room temperature ($\sim 20^\circ$) with 5000 rad of ⁶⁰Co γ -rays (dose rate 1000 rad/min). One-ml aliquots were distributed into culture tubes; incubation medium was added to give the same final concentration of serum and thymidine as in the experiments with other mutagens.

(c) *Chemical mutagens*. HN2 was supplied by Boots (Nottingham) and MMS by Kodak Ltd. (Kirkby, Liverpool).

The cells were suspended in saline and distributed into an appropriate number of 25-ml universal bottles depending on the number of doses used. The mutagen, dissolved in saline and diluted within 5 min prior to use, was then added at an appropriate concentration to give a total volume of 10 ml. After incubation for 1 h at 37°, the cells were removed from the treatment solution by centrifugation and washed twice in saline. They were then distributed for incubation.

Measurement of thymidine incorporation

Following mutagen treatment and subsequent washings, where appropriate, each sample (approx. $1-2 \cdot 10^7$ cells) was introduced into 3 ml Eagle's MEM medium containing 100 units/ml streptomycin, 100 units/ml Crystapen, and 10 or 20% foetal calf serum. Although less than 1 in 1000 peripheral blood lymphocyte cells are in S phase, $10^{-3}M$ hydroxyurea was added to suppress residual DNA synthesis¹⁰.

In the first series of experiments, [³H]thymidine (spec. act. 17–25 Ci/mM) was added as soon as the cultures were set up, to give a concentration of 10 μ Ci/ml; the cultures were incubated at 37° for various times up to 8 h post-treatment. Since in these experiments [³H]thymidine was available throughout the period between mutagen exposure and sampling, experiments of this type are referred to as "continuous labelling" experiments. For longer incubation times, 0.1 μ g/ml of cold thymidine was initially added to the medium and following a medium change [³H]thymidine usually introduced 2 h prior to harvesting the cells; such experiments are referred to as "pulse labelling" experiments.

After appropriate incubation times, the "hot" medium was spun off and the cells were washed in 3 changes of saline. 2 extractions with ice-cold 5% TCA were followed by a methanol wash and the residue was dissolved in "Soluene" (Packard Ltd.). Toluene with PPO and POPOP was added for counting in a Packard Tri-carb scintillation counter. The efficiency was estimated using an external standard, and was usually about 30%.

INTRODUCTION

Radiation-stimulated incorporation of labelled precursors into mammalian cell DNA was first shown by RASMUSSEN AND PAINTER⁴¹ in HeLa cells exposed to UV. This synthesis has been termed unscheduled DNA synthesis¹⁵ and is non-semiconservative^{12,42}, ³H-labelled thymidine entering both heavy (BUdR-labelled) and light strands of hybrid DNA in patches too small to affect its density. A main product of UV irradiation of DNA is the thymine dimer^{2,57} and the induced unscheduled synthesis is accompanied by DNA degradation resulting in excision of such dimers^{13,27,30,45}.

These observations on mammalian cells are analogous with those made earlier on bacteria^{3,40,51} which led to the idea of "excision repair", damaged single-strand regions being degraded and resynthesised using the complementary strand as a template. Recently, following exposure of mammalian cells to low doses of UV, "repaired" DNA has been shown to be able to undergo a normal semiconservative replication^{38,43}.

Unscheduled DNA synthesis has now been demonstrated in many mammalian cell types following exposure to UV light^{9,17-19,44}, X-rays^{22,37,42,53}, HN₂⁴⁷ and MMS^{1,48}. Removal of alkylated groups^{14,39,46} and radiolysis products²⁴ from DNA has also been demonstrated and it is assumed that the repair processes following alkylation and X-radiation have at least some steps in common with those observed after exposure to UV light.

Studies of the kinetics of unscheduled DNA synthesis following UV-irradiation of human lymphocytes by EVANS AND NORMAN^{18,19} have shown that the process lasts approximately 6 h at 37°. In a later study on X-irradiated lymphocytes, it was shown that after a similar 6-h incubation time the incorporation of DNA precursors was incomplete⁵².

We have been similarly interested in the process of unscheduled DNA synthesis in human lymphocytes and have compared the responses obtained following exposure to UV light, γ -rays, HN₂ and MMS. In the present report we confirm the difference between UV light and X-rays reported by SPIEGLER AND NORMAN and show that similar differences exist between the two chemical mutagens studied. The uptake of label into DNA following exposure to HN₂ was shown to parallel the results obtained with UV light whereas the pattern observed with MMS is similar to that following γ -irradiation. It is concluded that these similarities and differences in unscheduled DNA synthesis are a direct reflection of similarities and differences in the types of lesions induced in DNA by these mutagens.

MATERIALS AND METHODS

Preparation of leucocytes

Peripheral blood from polycythaemic or haemochromatotic patients was collected in citrate and used within 24 h. Leucocytes were separated as buffy coats following centrifugation at 1000 *g* for 15 min. After a second spin the cells were suspended in normal saline (Dulbecco's).

Mutagenic treatments

(a) *UV-irradiation.* 5-ml aliquots of resuspended leucocytes (approx. $5 \cdot 10^6$

UNSCHEDULED DNA SYNTHESIS IN HUMAN LEUCOCYTES AFTER EXPOSURE TO UV LIGHT, γ -RAYS AND CHEMICAL MUTAGENS

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SUMMARY

Unscheduled DNA synthesis in human peripheral blood lymphocytes has been compared following exposure of cells at various dose levels to UV light, γ -rays, methyl methanesulphonate (MMS) and nitrogen mustard (HN₂). Lymphocytes in G₁ were exposed to these mutagens and then incubated in a medium containing [³H]thymidine. For each mutagen the dose response and the temporal pattern of synthesis has been determined by scintillation counting of TCA (trichloroacetic acid)-extracted samples. In each case synthesis was found to initially increase with increasing dose, and following both types of radiation the initial increase was exponential. The system was found to be very sensitive to UV irradiation and unscheduled DNA synthesis has been detected at doses of 2 erg/mm², reaching a maximum at 40 erg/mm². The value obtained at dose levels above 40 erg/mm² was found to be constant for doses up to 5600 erg/mm² and then declined. Similar reductions in the amount of synthesis at high dose levels were also found with the chemical mutagens.

For estimations of the duration of the reaction, cells were harvested at various times up to 22 h post-treatment. A parallel response was found for UV- and HN₂-treated cells; for all doses considered, the reaction was largely completed within 6 h following exposure. This temporal pattern contrasted with the response following γ -irradiation and MMS treatment where synthesis continued for the whole of the incubation time considered. With γ -rays, the first 60 min following exposure was characterised by a phase of rapid synthesis, the amount of label incorporated increasing linearly over this period. The rate of incorporation between 1 and 22 h, however, although constant, was reduced to 25% of the early maximum rate. This biphasic response confirms and extends earlier studies and it is suggested that the early incorporation may be associated with the rejoining of radiation-induced single-strand breaks in the DNA. In the case of MMS a somewhat similar temporal pattern to that found with γ -rays was observed.

Consideration of the initial rates of the reactions following treatments with each of the mutagens, indicates that the unscheduled synthesis of DNA is an enzyme-limited reaction, possibly involving a different enzyme in the case of each mutagen.

Abbreviations: BUdR, 5-bromodeoxyuridine; HN₂, nitrogen mustard; MMS, methyl methanesulphonate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; TCA, trichloroacetic acid.