# DNA REPAIR DEFICIENCY IN LYMPHOCYTES FROM PATIENTS WITH ACTINIC KERATOSIS

by

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SUMMARY

1. The literature covering the relevant aspects of the photochemistry of nucleic acids and the biological effects of ultra violet light radiation on cells is reviewed. The DNA repair processes which have been shown to occur in micro-organisms and mammalian cells are discussed. The sun-sensitive conditions, xeroderma pigmentosum and actinic keratosis are described and compared. The defective DNA repair synthesis in cells from patients with xeroderma pigmentosum and the possible role of DNA repair defects in carcinogenesis are discussed.

2. Human peripheral blood lymphocytes were isolated and used, in preference to skin fibroblasts, to study ultra violet light-induced DNA repair in patients with different sun-sensitive conditions. The choice of lymphocytes for the study of DNA repair is explained.

3. Conditions for the study of ultra violet light-induced DNA repair in human lymphocytes by two different methods, were established. One method used autoradiographic analysis to follow ultra violet light-induced DNA repair synthesis in the non S-phase cells. The other measured the ultra violet light-induced incorporation of $[^3H]$ - thymidine into DNA of lymphocyte cultures in the presence of hydroxyurea, which blocks DNA replication in the small but significant fraction of*cells in the lymphocyte cultures.

* dividing

4. Results obtained by these two methods show that the rate of ultra violet light-induced DNA repair in lymphocytes derived from actinic keratosis patients is only 50% of that in lymphocytes from age-matched normal individuals after 4 hours (by which time the repair of DNA in normal lymphocytes is complete).

5. Actinic keratosis is a condition of elderly people (aged 50 years or over), so the effect of subject age on DNA repair was studied. It was shown that the rate of ultra violet light-induced DNA repair in normal subjects does not vary significantly in the age range 17 - 77 years.

6. The time course experiments indicated that, while the ultra violet light-induced DNA repair in normal lymphocytes is complete after 4 hours, that in actinic keratosis lymphocytes is not. Subsequently, it was shown that, given twice the time (8 hours), lymphocytes derived from actinic keratosis patients complete the same amount of repair as that furnished by lymphocytes derived from normal subjects.

7. It was shown that DNA synthesis, in the presence of hydroxyurea, following ultra violet light-irradiation of human lymphocyte cultures, is mostly due to DNA repair synthesis and not to some form of ultra violet light-induced DNA replication.

8. It was shown that the observed difference between DNA repair, measured by the incorporation of $[^3H]$ - thymidine in lymphocytes from actinic keratosis patients and normal subjects is not due to differences in $[^3H]$ - thymidine uptake or to different thymidine nucleotide pool sizes.

9. A novel method was developed to study DNA repair in lymphocytes, based on following the recovery of phytohaemagglutinin-stimulated DNA replication after inhibition by ultra violet light. This method, unlike those previously used to assess DNA repair, measures the extent of successful repair (in that it allows the continuation of DNA replication). The results obtained by this method confirmed that ultra violet light-induced DNA repair in lymphocytes derived from actinic keratosis patients is about 50% that of lymphocytes derived from age-matched normal individuals.

10. Preliminary experiments have shown that lymphocytes derived from patients with lupus erythematosus also have a reduced rate of ultra violet light-induced DNA repair, but that lymphocytes derived from patients with either melanoma or basal cell carcinoma have normal levels of repair.

11. The conclusions which have emerged from this study regarding the defective DNA repair in lymphocytes from patients with actinic keratosis are discussed. The possibility that

defective DNA repair is a common basis for both actinic keratosis and xeroderma pigmentosum is considered. The unlikely contribution of photoreactivation and post-replication repair mechanisms to the DNA repair measured in this work is discussed. The reasons in favour of actinic keratosis being genetically determined are considered.

# ABBREVIATIONS

## ABBREVIATIONS

The abbreviations recommended by the Biochemical Journal Instructions to Authors (revised, 1976) were used with the following additions:-

| | |
|---|---|
| AK | Actinic Keratosis |
| AS | Acid-soluble |
| BCC | Basal cell carcinoma |
| BrdUrd | 5-bromodeoxyuridine |
| BrUra | 5 bromouracil |
| BSS | Balanced salt solution |
| FdUrd | 5-fluorodeoxyuridine |
| LE | Lupus erythematosus |
| M | Melanoma |
| NL | Normal |
| OH-urea | Hydroxyurea |
| PBS | Phosphate buffered saline |
| PHA | Phytohaemogglutinin |
| PPO | 2, 5 diphenyloxazole |
| PYPY | Pyrimidine dimer |
| SDS | Sodium dodecyl sulphate |
| TCA | Trichloroacetic acid |
| U.V. | Ultra violet |
| XP | Xeroderma pigmentosum |

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# 1. INTRODUCTION

## 1.1 Biological effects of Ultra Violet (u.v.) Radiation

The first observation of u.v. effects on living systems was made by Downes and Blunt (1877), who reported that bacteria were inactivated by light. Gates (1928) found that the relative effectiveness of killing bacteria by different wavelengths paralleled the absorption spectrum of nucleic acids. A decade later, the similarity between the action spectrum for u.v. mutagenesis and the absorption spectrum of deoxyribonucleic acid (DNA) was reported by Emmons and Hollaender (1939) and by Knapp et al (1939).

The relationship between the development of human skin cancer and u.v. radiation is derived from clinical impressions and the epidemiology of the condition (Blum 1959; Urbach et al 1966). An understanding of the carcinogenic potential of u.v. radiation comes from studies on laboratory animals (Roffo 1934; Epstein 1970; Emmett 1973). Direct evidence implicating u.v. radiation with neoplastic transformation has been presented by Hart and Setlow (1975) and Hsu et al (1975).

Although most of the studies of biological effects of u.v. radiation have used u.v. light of specific wavelengths (i.e. experiments using germicidal lamps with principal output of 254 nm), there is evidence that similar effects can be produced by radiation of longer wavelengths including those found in sunlight (Harm 196[illegible]).

It was estimated by Hahm (1969) that the average germicidal acitivity of sunlight which is essentially due to wavelengths near 300 nm on a clear day between 9 a.m. and 12 a.m., is equivalent to 0.1 $J.m^{-2}.min^{-1}$ of 254 nm u.v. radiation. This was later confirmed by Trosko et al (1970) who demonstrated that normal exposure to natural sunlight which contains no detectable wavelengths shorter than

290 nm at sea level, can give rise to a significant formation of thymine dimers in human cells grown in vitro. Thymine dimers being the main photoproduct of u.v. irradiated DNA (see section 1.3.3) and are directly involved with neoplastic transformation (Hart and Setlow 1975).

### 1.2.1 DNA is the most sensitive target for ultra violet radiation

By virtue of the high extinction coefficients of its constituent basis in the u.v. region, its importance to the duplication of the cell, and its sensitivity to change in terms of precision of function, DNA is the most sensitive target for the damaging effects of u.v. photons on cells. Ribonucleic acid (RNA) may be similarly sensitive to some types of photochemical damage, but the result is not as drastic because of the multiplicity of identical RNA molecules which can be replaced using the genetic information of DNA. For similar reasons, and also because of the much smaller extinction coefficients, protein inactivation by u.v. light will not generally be important in biological effects.

### 1.2.2 Evidence showing that DNA is the biologically important target for u.v. irradiation

(1) Action spectra

Gates (1930) showed the rate of killing bacteria with u.v. light is maximum at 260 nm. This seems to be in fair agreement with the nucleic acid absorption spectrum (Hollaender and Emmons 1941).

The u.v. absorption spectrum of nucleic acids shows a well defined peak at 259 nm. which is due to absorption by the chromophoric groups of the purines and pyrimidines. The extent of the absorption at this wavelength is dependent not only upon the pH and ionic strength of the solution, but also upon the previous treatment of the nucleic acid sample.

Due to the variable water content of nucleic acids, the extinction coefficients can not be based on the weight of the nucleic acid since the "dry" weight has little significance. However, nucleic acids contain one phosphorus atom for every purine or pyrimidine molecule which led Chargaff and Amenhoff (1948) to suggest that the absorption of a nucleic acid solution should be expressed in terms of the extinction per gramme-atom of phosphorus per litre.

Proteins exhibit selective absorption in the u.v. region of the spectrum with a maximum of about 280 nm and a minimum of 250 nm. Hence the u.v. absorption spectrum for proteins does not coincide with the u.v. killing spectra. Figure 1 shows the relative u.v. absorption measured spectrophotometrically on sections of non-dividing interphase tissue cell from mammalian liver (Caspersson 1950).

(2) Nuclear or Cytoplasmic irradiation of Habrobracon eggs

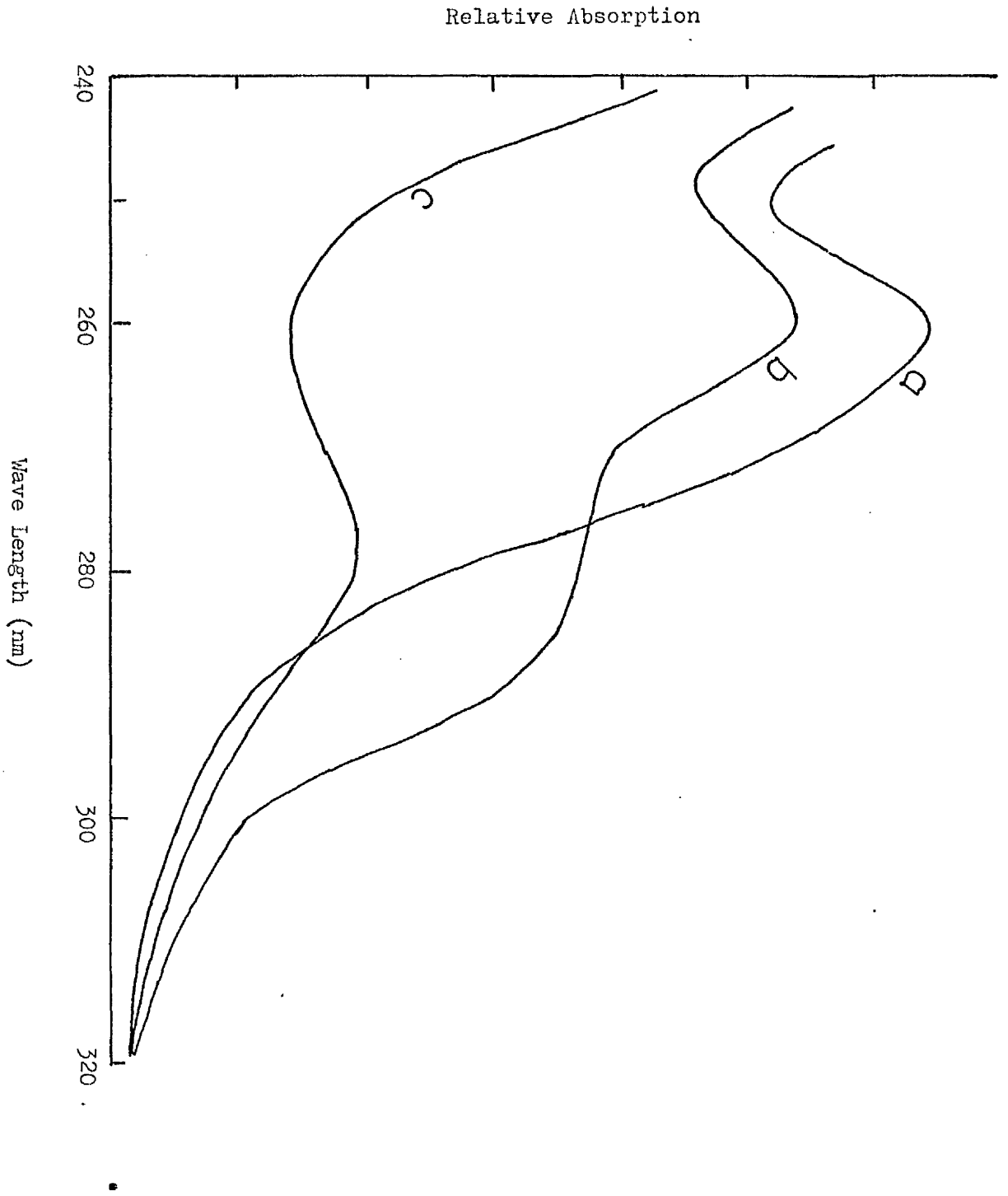
By shielding either the nucleus or the cytoplasm of eggs of the wasp Habrobracon exposed to u.v. radiation, Von Borstel and Wolff (1955) showed that damage to the nucleus resulted in a much greater reduction in survival as measured by hatchability.

The egg of the wasp Habrobracon has characteristics that makes it possible to irradiate the nucleus and cytoplasm separately. Fertilization of the egg is not required for normal development, unfertilized eggs become haploid males. The nucleus of the newly laid egg is in the meiotic metaphase and is located at the anterior. It remains in this position for 30 mins at 30°C while meiosis is completed. The nucleus then migrates towards the centre of the egg for development to begin.

(3) Ultra violet microbeam irradiation of tissue cells

U.v. micro irradiation can also be used to perturb or destroy different organelles or parts of a living mammalian cell, leaving the remainder relatively unaffected (Zirkle 1957; Smith 1964; Moreno et al 1969).

Fig. 1. The u.v. absorption spectra for (a) nucleic acids (b) nucleolus (c) protein, in a non-dividing interphase tissue cell from mammalian liver. Data obtained from Caspersson (1950).


Relative Absorption
240
260
280
300
320
Wave Length (nm)
C
B
A

In this way a microbeam can be used to study the effects produced in a restricted area of the nucleus and the unirradiated part of the same nucleus remains as a control.

Moreno (1971) partially irradiated nuclei of human (KB) cells with a 5 nm diameter microbeam of u.v. light (275 nm). KB cells were originated from the culture of a biopsy material from poorly differentiated epidermoid carcinoma, developed by a man named KB. A dose of approximately 100 $J.m^{-2}$ did not produce any morphological changes that could be discerned by electron microscopy, but it did inhibit mitosis for at least 24 h. When the same cells were incubated with tritiated thymidine ($[^3H]$ - dT) for 3 h, the radioautograms showed the nucleoside was incorporated into the irradiated zone. The same dose of irradiation applied to cells normally undergoing DNA synthesis (S - phase), gave rise to the appearance of label throughout the nuclei of these cells. However, over 40% of these cells showed a reduced incorporation of $[^3H]$ - dT within the irradiated spot, as compared with the non irradiated part of the nucleus.

(4) The sensitizing effect of bromouracil

Cells or bacteriophage in which bromouracil (BrUra) is substituted for thymine in the DNA are considerably more sensitive to u.v. killing (Greer and Zamenhoff 1957; Stahl et al 1961). Greer (1960) showed that thymine requiring E. Coli $15T^-$ and E. Coli $B^-$ which contain BrUra in their DNA instead of thymine have a marked increase in their sensitivity to u.v. radiation. The effect was proportional to the extent of incorporation of BrUra under defined growth conditions and for a given medium. There was as high as a 20,000 fold decrease in survival as compared to irradiated bacteria grown in the absence of the analogue. There was no increase in u.v. sensitivity in organisms grown in the presence of fluorouracil (FUra). Since FUra is incorporated into RNA and not into

DNA (Chaudhuri et al 1958) and BrUra is incorporated into DNA (Dunn and Smith 1954), it follows that DNA rather than RNA must be the more important target for the lethal effects of u.v. radiation.

The difference between the u.v. absorption of thymine and that of BrUra may be responsible for the sensitization effect of the analogue. Smith (1964) reported that BrUra containing DNA exhibited a much greater sensitivity to u.v. induced cross linking with proteins.

(5) U.V. inactivation of Haemophilus transforming DNA

Setlow and Setlow (1962) have shown that the in vitro rate of inactivation of Haemophilus transforming DNA by u.v. irradiation correlates with the appearance of thymine dimers in the acid soluble fraction. Thymine dimers being the main photoproduct of u.v. irradiated DNA (see section 1.3.3).

1.3.1 Photochemistry of the nucleic acids

While the damaging effects of u.v. radiation on living organisms have long been known at the biological level, it is only recently that the photochemical bases for many of these effects have been elucidated.

The correlation between the survival of u.v. irradiated cells and the production of certain types of identifiable photochemical damage in their DNA, has gained impetus since the discovery by Beukers and Berends (1960) that irradiation of thymine in frozen aqueous solution gives rise to a cyclobutane dimeric product.

During the last 15 years, the study of the photochemistry of nucleic acids and their constituents has become a very active area of research. There are numerous reviews by various authors dealing with this subject (Smith 1964; Burr 1968; Setlow 1968; Hanawalt 1968; Smith and Hanawalt 1969; Harm et al 1971).

1.3.2 Effect of u.v. radiation on nucleic acid constituents

The components of nucleic acids are purines, pyrimidines and sugar phosphates. Since sugar phosphates do not absorb u.v. above 220 nm to any significant level, they are unaffected by biologically effective wavelengths of u.v. radiation. From quantum yield considerations, the purine bases are 10 times less sensitive to u.v. than pyrimidine bases (Smith and Hanawalt 1969).

Hence a major fraction of the lethal and mutagenic effects of u.v. radiation on biological systems has been attributed to photochemical transformations of pyrimidine residues (McLaren and Shugar 1964). Depending on the irradiation conditions and the environment during irradiation, pyrimidine bases form different products (Smith 1967).

1.3.3 Pyrimidine dimers

The chain of inferences relating pyrimidine dimers to biological lesions depends not only on the identification of such dimers in u.v. irradiated DNA, but also on the demonstration that the dimers and not some other photochemical event, are responsible for the observed biological effects.

Thymine dimers have been identified as the principal product resulting from the u.v. irradiation of thymine in frozen aqueous solutions by molecular weight determinations, x-ray diffraction, infra red absorption spectroscopy and molecular magnetic resonance techniques (Beukers and Berends 1960).

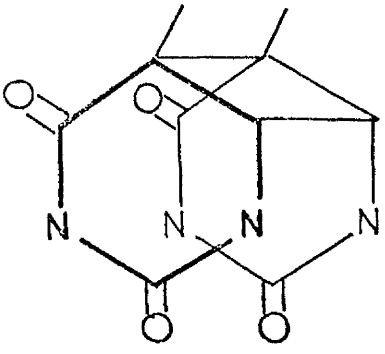
Photodimerization of a number of $\alpha,\beta$-unsaturated keto derivatives has been observed (Cohen and Schmit 1964). Thymine and uracil exist predominantly in the keto form (Jordon 1955; Mason 1957; Miles 1961). This is because the keto tautomer has the lowest energy (Mason 1958). As expected, the u.v. irradiation of thymine and uracil in aqueous

solution and in the presence of a suitable photosensitizer produces dimers in excellent yield (Lamola 1969). Since cytosine lacks the $\alpha, \beta$ unsaturated keto group of thymine and uracil it has been suggested (Wang 1961) that the small amounts of uracil dimers formed when cytosine is irradiated in frozen solution (Smith 1963; Wacker 1963) might be due to the deamination of cytosine prior to dimerization. However, according to Greenstock and John (1968) cytosine does not form dimers by direct or acetone sensitized irradiation, although Varghese (1972a) has shown that cytosine dimerizes in poor yield when irradiated in solution at a wavelength greater than 290 nm in the presence of acetone.

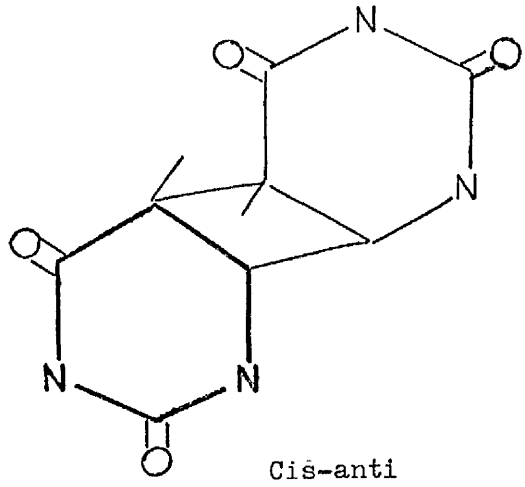
Formation of cyclobutane type dimers from the addition of two pyrimidine residues at the C5 - C6 double bonds, is the most extensively studies photochemical reaction (Smith 1964; Setlow 1968; Johns 1971). Jennings et al (1970) have isolated the four possible isomers of the thymine dimers (Fig. 2), previously postulated by Wulff and Fraenkel (1961) from acetone-sensitized u.v. irradiated thymine. These are the Cis-syn, Cis-anti, Trans-syn and Trans-anti. The predominant form isolated from irradiated DNA is the Cis-syn isomer.

The different isomers of a particular dimer are usually identified on the basis of their infra red absorption spectra, chromatographic mobilities and stability in acidic and alkaline solutions (Weinblum and John 1966; Varghese 1971). Cyclobutane pyrimidine dimers show only end absorption in the 260 nm u.v. region (Fig. 3), but are converted to the monomers, thus regaining the 260 nm absorption band after irradiation at 254 nm or shorter wavelengths in aqueous solution, and are often identified on the basis of this property.

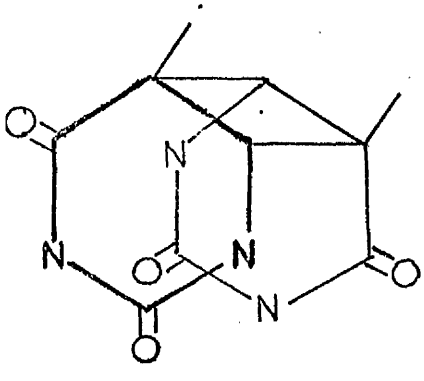
Fig. 2 The four possible isomeric structures of the (cyclobutane type) thymine dimer, predicted by Wulff and Fraenkel (1961).



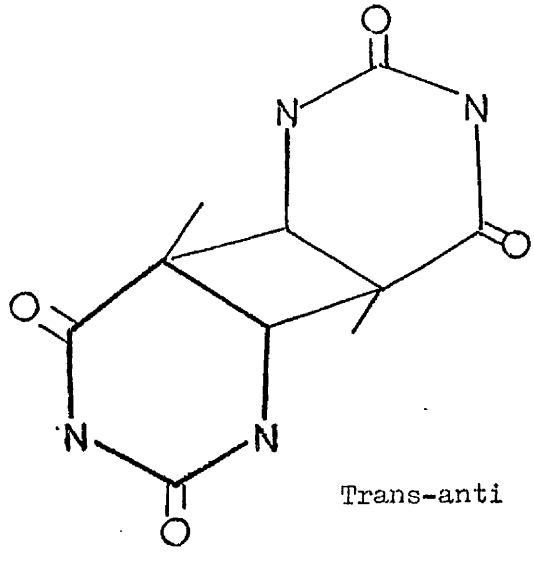
Cis-syn



Cis-anti

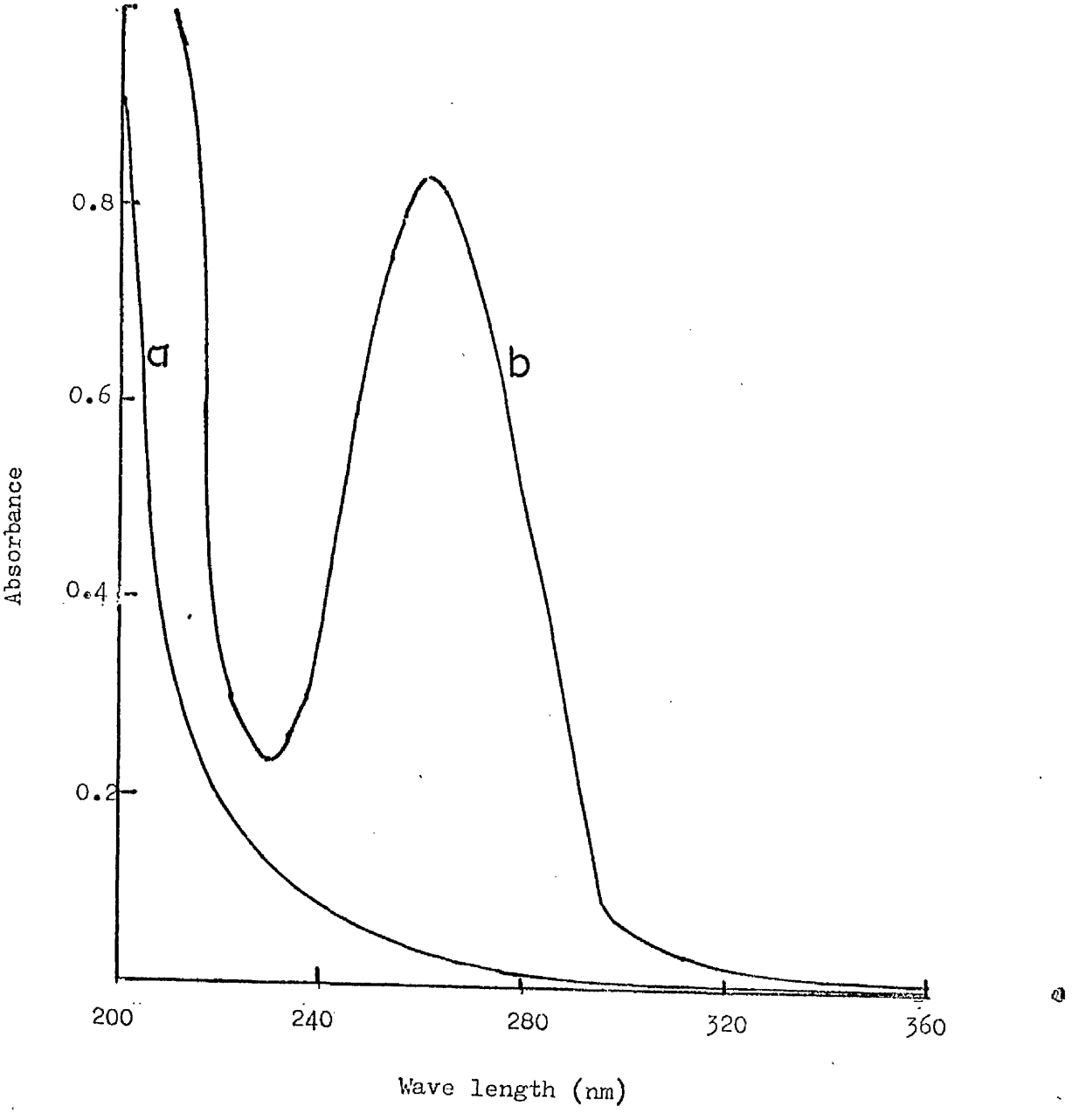


Trans-syn



Trans-anti

Fig. 3 Ultra violet absorption spectra of cyclobutane-thymine dimer (a) in aqueous solution;
(b) after exposure to 254 nm radiation.
Data is given by Varghese (1972b)


Absorbance
0.8
0.6
0.4
0.2
a
b
200
240
280
320
360
Wave length (nm)

The formation of cyclobutane pyrimidine dimers is a photochemically reversible reaction (Setlow 1968) as indicated in Equation 1.

$$PY\ PY \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \overline{PY\ PY} \qquad \ldots\ldots (1)$$

where PY PY are the adjacent pyrimidine residues

$\overline{PY\ PY}$ is the cyclobutane pyrimidine dimer

$K_1$ is the rate constant for the forward reaction

$K_2$ is the rate constant for the reverse reaction

$K_1$ and $K_2$ are proportional to the intensity of the incident light, the absorption coefficient and the quantum yields. The absorption coefficient and the quantum yield are defined as the probability of an incident quantum being absorbed and the probability that an absorbed quantum produces a chemical change (Setlow 1966). The absorption coefficient, quantum yield and therefore the steady state concentration of dimers depend upon pH and temperature (see Fig. 4 ). Formation of dimers is favoured in irradiated frozen solutions, since in this case the molecules are held close together in the form of solid aggregates (Wang 1961). This results in a higher probability of an absorbed photon forming a dimer (quantum yield for the formation of thymine dimer in frozen solution is between 1 and 2). In dilute liquid solution, by contrast, where the molecules are much farther apart, the quantum yield for dimer formation is zero while that for splitting dimers is between 0.5 and 1 (Setlow 1961).

Saturation of the C5 - C6 double bond of pyrimidines leads to characteristic changes in their absorption spectra (see Fig. 4 ).

Fig. 4 The absorption spectra of thymidine, thymine dimer, cytidine and dihydrocytidine, given by Setlow (1966). a and b show the effect of pH on the absorption curves of thymidine and thymine dimer.

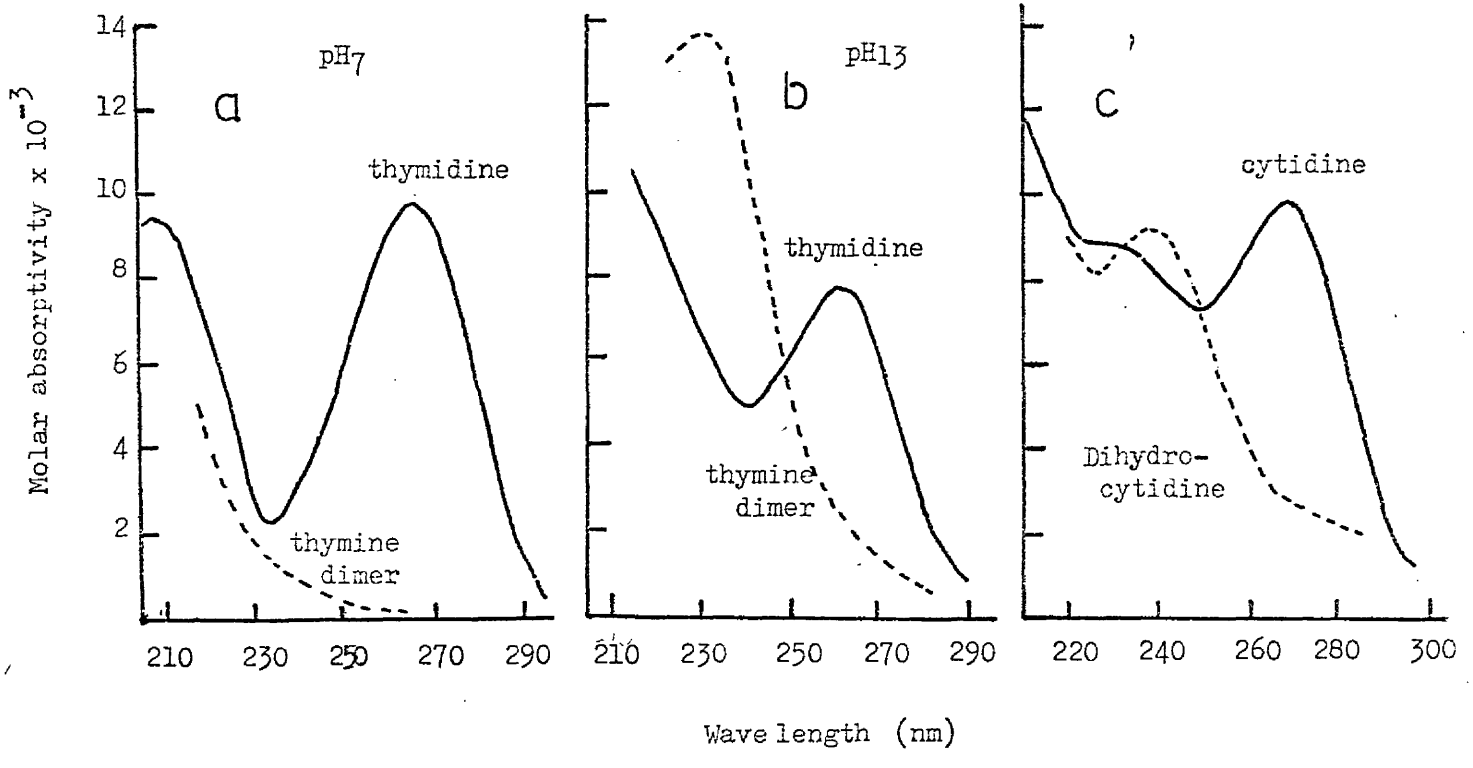

Molar absorptivity x $10^{-3}$
14
12
10
8
6
4
2
a
pH7
thymidine
thymine
dimer
210
230
250
270
290
b
pH13
thymidine
thymine
dimer
210
230
250
270
290
c
cytidine
Dihydro-
cytidine
220
240
260
280
300
Wave length (nm)

Fig. 4 shows that pyrimidine dimers absorb u.v. light at much shorter wavelengths than do their corresponding monomers. Hence the steady state concentration of the dimers is wavelength dependent. Thus irradiation with long wavelengths of u.v. light (e.g. 260 nm) favours the formation of dimers resulting in a decrease in absorbance, whereas short wavelengths (e.g. 230 nm) will split some of the dimers and cause a subsequent increase in the absorbance (Setlow 1966, 1968).

Not long after the identification of thymine dimer as the irradiation product of frozen solution of thymine, it was shown that the same product can be isolated from acid hydrolyzates of u.v. irradiated DNA (Beukers et al 1960; Wacker et al 1960). Varghese and Wang (1967) isolated the dimeric product from calf thymus DNA and identified it as the Cis-syn isomer. According to Ben-Hur and Ben-Ishai (1968), a second isomer of thymine dimer is formed as a minor product from irradiated denatured DNA. Based on chromatographic mobilities, it has been suggested that the minor dimer is the Trans-syn isomer. No evidence for the formation of the other two isomers of thymine dimer in DNA has been reported.

Cytosine-cytosine and cytosine-thymine dimers are formed in native DNA (Setlow and Carrier 1966). These dimers undergo easy deamination, forming uracil-uracil and uracil-thymine dimers respectively.

Products formed in the DNA of u.v. irradiated bacteria appear to comprise 50% thymine-thymine, 40% thymine-cytosine and 10% cytosine-cytosine (in the form of thymine-uracil and uracil-uracil respectively) dimers. The yield of pyrimidine dimers in bacterial DNA is approximately $2.6 \times 10^{-6}$ dimers per thymine residue per 0.1 $J.m^{-2}.sec^{-1}$ at 254 nm (Setlow 1967; Howard-Flanders 1968).

Pyrimidine dimers induced by u.v. light are normally formed between adjacent pyrimidine residues in the same strand. Inter-strand dimers can not be formed in native DNA, since their base pairing contradicts the complementarity of purine-pyrimidine base pairing suggested by Watson and Crick (1953); also the individual pyrimidine residues in each strand are held firmly in their confirmations by hydrogen bonding and base stacking. However, interstrand dimers may be formed in u.v. irradiated DNA which is partially denatured (Marmur and Grossman 1961).

Due to the helicity of the double stranded DNA, the formation of a pyrimidine dimer in the same strand will necessarily be accompanied by some strain on the cyclobutane ring. The least strain is needed in the formation of Cis-Syn dimer.

It is now clear (see Section 1.3.3) that u.v. induced pyrimidine dimers and in particular thymine dimers, are the main cause of the lethal effects of u.v. irradiation, and Hart and Setlow (1975) have recently produced direct evidence that u.v. induced pyrimidine dimers in DNA can result in neoplastic transformation.

### 1.3.4 Photohydration of Pyrimidines

There has recently been an increase in interest of photohydrates of pyrimidines, which are formed by water addition across the C5 - C6 double bond.

A study of the pH and temperature dependencies of photohydration of several pyrimidines (Burr et al 1972) as well as viscosity effects on this reaction (Summers and Burr 1972) have led to the conclusion that the reactive state is an excited singlet state of the neutral species (pyrimidine molecules) and not a protonated excited species,

as suggested earlier by the same authors. The rate of hydrogen deuterium exchange at the C5 position of uracil derivatives in acidic media is enhanced by u.v. irradiation as a result of water addition at the 5 - 6 double bond (Ransworth et al 1972). Chattak et al (1972) have also reported that u.v. irradiation of uracil and several of its derivatives in "acid puddles" formed in frozen acidic aqueous solutions leads to high yields of 6 - hydroxy - 5, 6 - dihydrouracil.

The rate of photohydration of uracil residues in poly (U) is identical to that of UMP, while the photohydration of poly (C) follows biphasic kinetics (Lomant and Fresco 1972a). Due to stacking of cytosine bases in poly (C), the initial rate of cytidine photohydration is decreased as compared to CMP. Photohydrate formation induces a de-stacking which leads to an increase in the photohydration rate. Non-complementary uracil residues in poly (A,U) poly U double helices are fully accessible to solvent molecules and have an unstacked conformation, as revealed by a photohydration rate identical to that of UMP (Lomant and Fresco 1973). Lomant and Fresco (1972b) have reviewed these aspects of u.v. photochemistry that can be used to probe polyribonucleotide conformation.

U.V. inactivation and miscoding of u.v. irradiated R17 coliphage single-stranded RNA have been ascribed to pyrimidine photohydrate formation (Remsen and Cerutti 1972). Unstable cytidine photohydrates can be converted to a stable reduction product by sodium borohydride treatment (Mattern et al 1972). Cytidine photohydration occurs with 10 - 15 times lower efficiency than uridine photohydration in R17 RNA. Uridine photohydrates represent the main lethal lesions. Taking advantage of the fact that the R17 coat protein usually lacks histidine,

Remsen and Cerutti (1972) have shown that uridine photohydrates can miscode as cytidine in an in vitro translation system.

The stability of cytidine photohydrates in DNA is such (half-life of 58 min in native *E.coli* DNA at pH 8.1 and 20°C) that they must be considered as lesions of possible biological importance (Vanderhoek and Cerutti 1973).

1.3.5 Protein - DNA cross linking

Smith (1962) studied the effect of increasing doses of u.v. on the amount of DNA essentially free of protein that can be extracted from bacteria (unirradiated controls as 100%). 30% of the DNA is about 7 times more sensitive to the u.v. than the remainder. The amount of the free DNA that is lost from the supernatant in their assay due to irradiation can be quantitatively accounted for in the detergent-protein precipitate. At 99% killing of *E.coli* B/r, i.e. 180 $J.m^{-2}$, only 0.1% of the thymine in the DNA is converted to the thymine dimers (Smith 1962), yet the same dose renders 11% of the DNA unextractable. However, the biological significance of protein - DNA cross linking is not yet understood.

1.3.6 Photochemical reactions of purines

It has been generally assumed that purine bases play no role in the biological effects of u.v. irradiation. However, recent experiments have demonstrated that u.v. irradiation of DNA in the presence of alcohols such as 2-propanol lead to hydroxy-alkylation of purine bases at the C(8) position, while pyrimidine dimer formation is decreased (Ben-Ishai *et al* 1973). Photochemical reactions of nucleic acid constituents with 2-propanol initiated by light of

wavelength greater than 290 nm in the presence of di-t-butyl peroxide are selective for purines (Leonov et al 1973).

Since the sensitivity of the purine bases toward u.v. radiation is one-tenth that of the pyrimidine bases (see section 1.3.2), by the time a significant purine damage has occurred, the cells would have been inactivated by pyrimidine damage.

### 1.4.1 The repair of radiation damage

The first indication that cells might have the capacity to recover from radiation damage was the observation that minor modifications in the handling of the cells, e.g. growth media, temperature, etc., had a marked effect upon the ultimate viability of irradiated cells. Thus Hollaender and Claus (1937) found that higher survival levels of u.v. irradiated fungal spores could be obtained if they were allowed to remain in water or salt solution for a period of time before plating them on a nutrient agar. Roberts and Aldous (1949) extended these observations by showing that the shapes of the u.v. survival curves for E.Coli B could change drastically simply by holding the irradiated cells in media devoid of an energy source for various times before plating on nutrient agar. This phenomenon, known as liquid holding recovery, has been shown to require intact uvr genes (Ganesan and Smith 1969), that control the first step (Howard-Flanders et al 1966) in excision repair (section 1.4.2).

The most extensively studied repair process in micro organisms are: photoreactivation, excision repair and recombination repair.

1.4.2 Photoreactivation

"Unless otherwise specified u.v. implies the wavelength region 220 - 300 nm".

Photoreactivation can be defined in a broad sense as the reduction in response to u.v. irradiation (at wavelengths greater than 310 nm) of a biological system resulting from a concomitant or post-treatment with non-ionising radiation (Jagger and Stafford 1965). Such an effect was described in the alga, Fucus furcatus by Whitaker (1941) and was possibly seen even earlier (Prat 1936), but recognition of photoreactivation as a general phenomenon stems from its rediscovery by Kelner (1949) in bacteria and almost simultaneously by Dulbecco (1949) in bacteriophage. Since then, photoreactivation effects have been shown to range very far through the biological world.

Enzyme-catalysed photoreactivation is the most important and most thoroughly characterised repair mechanism. Such mechanism requires light energy in the near u.v. and violet-blue spectral range for its action (i.e. wavelengths ranging from about 310 - 480 nm).

Indications of the involvement of enzymatic processes in photoreactivation were first obtained when Dulbecco (1950) observed that photoreactivation of phage occurs only if the light is applied to intracellular phage, while the inactivating u.v. radiation can be applied to either the extracellular or the intracellular phage. No photoreactivation was observed when the phage alone, or the host cell alone, or when both independently, were illuminated. This suggested that, besides the light, a cellular factor is required. Investigation of the photoreactivation kinetics in phage by Dulbecco (1950) and Bowen (1953) further indicated the involvement of a temperature-

dependant dark reaction, suspected to be enzymatic in nature.

Real evidence for the requirement of an enzyme in the photoreactivation process was provided by *in vitro* studies, using mixtures of u.v. irradiated transforming DNA of *Haemophilus influenzae* and extracts of *E.coli* cells (Goodgal *et al* 1957; Rupert *et al* 1958). If such mixtures were exposed to photo-reactivating light (wavelength greater than 310 nm), the u.v. survival of transforming activity was increased. Exposure of the DNA alone, or the extract alone, or both separately to photo-reactivating light, did not increase the transforming activity.

Elaboration of this *in vitro* photoreactivation system, using yeast extracts (Rupert 1960, 1961, 1962) demonstrated the existence of a photoreactivating enzyme in the *E.Coli* extract.

Photochemical investigations showed that cyclobutyl pyrimidine dimers in u.v. irradiated DNA are the photo-repairable lesions. In the presence of photoreactivating enzyme, the cyclo-butane pyrimidine dimers monomerize when exposed to near u.v. or visible light illumination (Rupert 1964; Cook 1967). The kinetics of this enzymatic reaction have been investigated (Harm *et al* 1971). The evidence produced by Williams *et al* (1971) that a synthetic decanucleotide $(pT)_4$ pTpT $(pT)_4$ in which a Cis-syn thymine dimer residue has been incorporated in the central position, is a substrate for the photoreactivating enzyme, clearly suggests that thymine Cis-syn dimers in DNA are substrates for the enzyme. Cyclobutane dimers of cytosine and uracil also function as substrates, though they are monomerized at lower rates (Setlow and Carrier 1966).

Photoreactivation (restoration of the transforming ability of

u.v. inactivated transforming DNA) with the yeast extract system follows the classical enzyme kinetics. The enzyme is mechanically bound to u.v. irradiated DNA in the dark, but not to unirradiated DNA, as judged by the ability of irradiated DNA to protect the enzyme against inactivation by heat and heavy metals (Rupert 1962). The absorption spectrum of the purified enzyme, which is different from the action spectrum for the in vitro photoreactivation, indicates the lack of chromophore. This might mean that either the chromophore was destroyed during purification, or the chromophore occurs as a consequence of the complexing of enzyme and DNA (Setlow 1967; Smith and Hanawalt 1969).

Experiments with u.v. inactivated transforming DNA have confirmed that photoreactivation involves monomerisation of dimers, by showing that enzymatic photoreactivation overlaps that produced by short wavelength reversal (see Section 1.3.3). Moreover, the rate of reactivation of the transforming ability was found to be decreased in the presence of certain irradiated polynucleotides, which compete with irradiated transforming DNA for the photoreactivating enzyme. This competition was eliminated by prior illumination of the irradiated polymers in the presence of enzyme extracts (Setlow 1967; Setlow 1968).

Photoreactivating enzymes have been found in a wide variety of organisms, such as bacteria, moulds, sea urchin, fish, chick embryos (Setlow 1968) and plants (Trosko and Mansur 1969). They have also been recently isolated and characterized from human leucocytes (Sutherland 1974) and from cultured human fibroblasts (Sutherland et al 1975).

The demonstration that human cells possess photoreactivating enzyme implies that a direct test by photoreactivation may be made of the role of pyrimidine dimers in the induction of abnormal cell growth.

1.4.3 Excision repair

All cells, both bacterial and eucaryotic, appear to have enzyme systems which can recognise distortions caused by u.v. radiation in the DNA double helix and respond by excising them and synthesising a replacement stretch of DNA. The degree of resistance to u.v. radiation depends upon the repair efficiency of that particular organism. Only the enzymes of *E. coli* and *Micrococcus* have been characterized in any detail. *Micrococcus* is especially efficient in removing thymine dimers from DNA and can tolerate large doses of u.v. irradiation; *E. coli* on the other hand offers the advantage that a large number of mutant bacteria have been isolated which lack the ability ( or have reduced ability) to overcome irradiation damage so that their properties can be compared with more resistant strains.

Many genetic loci which are concerned with determing the degree of sensitivity of *E. coli* to radiation and mutation have been mapped and identified in both $K_{12}$ and B strains (Witkin 1967; Hanawalt 1968).

Phages which do not genetically specify their own repair systems make use of those of their bacterial hosts and the ability of bacteria to repair damaged phage DNA can be used as an assay for the host correction systems. Bacteria which lose the ability to repair damage to the DNA of infecting phages are described as *hcr*;

(host cell reactivation). Mutants of the hcr phenotype show a 10 to 20 fold increase in sensitivity to both the lethal and mutagenic effects of u.v. irradiation.

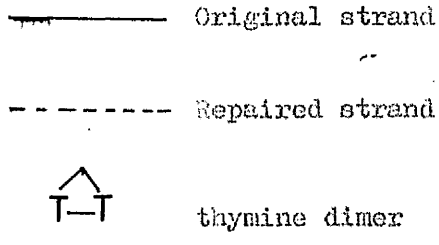
There is a high correlation between the survival of colony forming ability in u.v. irradiated cells and their ability to sustain plaque formation when the cells are used as hosts for irradiated phages (Lewin 1974). A second mutant phenotype, originally characterized by its sensitivity to u.v. light, because the cells can not repair damage in their own genomes are described as uvr. It is now clear that uvr and hcr describe the same genotypes.

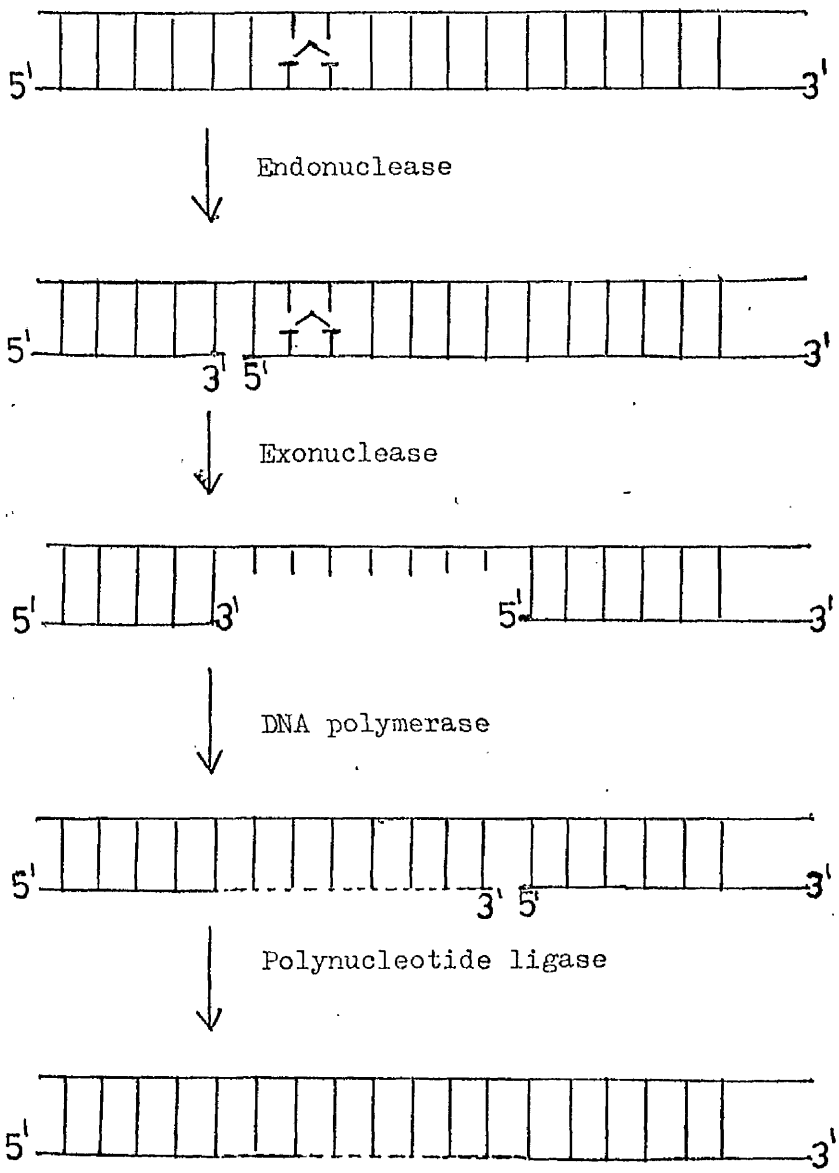
The uvr system is responsible for removal of thymine dimers from DNA by a mechanism which involves excision of a stretch of the single strand of DNA containing the dimer and its replacement by synthesis of a new strand. Howard-Flanders et al (1966) reported that mutation in any one of three unlinked loci, uvr a, b, c, causes inability to remove thymine dimers from DNA by this mechanism. This type of repair mechanism is known as dark repair, DNA repair, replication or excision repair.

The general features of the excision repair may be summarised as follows (see Fig. 5):

1. Pyrimidine dimers are recognized by an endonuclease (absent in uvr mutants), which introduces a single-strand break near the site of the damage.
2. Pyrimidine dimers and adjacent nucleotides of the damaged strand are excised by an exonuclease.
3. The excised region is repaired by a polymerase (absent in Pol A mutants), which uses the intact strand as template.
4. The intrastrand gap is closed by a ligase (absent in lig mutants).

Fig. 5 Schematic representation of the excision repair process, originally proposed by Howard-Flanders and Boyce (1966)




5'
3'
Endonuclease
5'
3'
3'
5'
Exonuclease
5'
3'
5'
3'
DNA polymerase
5'
3'
3'
5'
Polynucleotide ligase
5'
3'

Enzymes which are associated with the incision aspect of the excision repair have been isolated from both E. coli (Braun et al 1975) and Micrococcus luteus (Kaplan et al 1969). These enzymes are called Correndonucleases. Correndonucleases are defined as endonucleases whose specificity eventually leads to correctional mechanisms and which is limited to a DNA whose structure is modified such that its nitrogenous bases are no longer complementing, are removed from the DNA, or have interacted or can interact with bases in the same strand (intrastrand dimers) or have interacted with bases on the opposite strands (interstrand dimers, crosslinks) (Grossman 1975). The correndonucleases isolated from M. luteus and E. coli have been shown to act specifically on the strand damaged by pyrimidine dimers (Radman 1976). The use of correndonucleases for identifying pyrimidine dimers has provided a fairly sensitive and specific tool for measuring the removal of pyrimidine dimers in vivo (Ganesan 1974).

The removal of the damaged nucleotides from DNA can proceed by two alternate and perhaps interdependent routes (Grossman 1975). In addition to its polymerising properties, E. coli polymerase I has associated exonuclease activities which are capable of two sorts of repair processes. A $5' \rightarrow 3'$ associated double-strand-specific exonuclease is capable of removing pyrimidine dimers in its path concomitant with polymerisation. Furthermore, when non-complementary nucleotidesare incorrectly inserted during polymerisation, the $3' \rightarrow 5'$ single-strand-specific exonuclease is capable of digesting in a direction opposite of that of polymerization to the point of the first stable hydrogen bond (Brutlag and Kornberg 1972; Muzyczka et al 1972). Hamilton et al (1974) rule out the associated $3' \rightarrow 5'$ exonuclease

activity in the excision process. However, the $5' \rightarrow 3'$ exonucleolytic activity of DNA polymerase I enzymes suitably assume the excision role in this repair pathway. The ability of this $5' \rightarrow 3'$ exonuclease activity to hydrolyse non terminal phosphodiester bonds, by-passing photoproducts, renders this activity suitable for repair of this type. Most of the evidence, obtained from studies on M.luteus and E.coli correndonucleases, is that the phosphodiester bond incised is no more than one nucleotide away from the pyrimidine dimer (Kushner et al 1971), therefore, the $5' \rightarrow 3'$ exonucleolytic activity is capable of circumventing such damage during polymerization. The extent of pyrimidine removal depends on the relative rates of excision and polymerization which provide 3' hydroxyl forming appropriately juxtaposed for ligase action to the 5' termini destined for removal during the nick-translation process (Cozzarelli et al 1969).

Finally the possible participation of polynucleotide ligase in the early steps in the excision pathway is augmented by its involvement in the final steps of the preparation of fully active DNA. The specificity of this enzyme requires a 5'-phosphoryl group next to a juxtaposed 3'-hydroxyl group, for end-to-end joining (Sgaramella et al 1970). Furthermore, DNA containing non complementary nucleotides at its 3'-end is also a suitable substrate for the ligase (Tsiapalis and Marang 1970). Conformational changes associated with some pyrimidine dimer containing regions are obviously not too extreme for this enzyme to function. The ligase, therefore may assume, in addition to sealing the final phosphodiester bond, a level of control at earlier steps in the excision repair process.

The importance of excision repair mechanism is manifested in the enhanced survival of u.v. irradiated resistant stains of E.Coli, as compared to the excision defective mutants. Thus experiments conducted by Setlow and Carrier (1964) and Boyce and Howard-Flanders (1964), have shown that although u.v. light induces the same amount of thymine dimers in both resistant and sensitive cell types, the disappearance of dimers from the acid insoluble fraction and their appearance in the acid soluble is observed only in the resistant strain but not in the uvr mutants.

Evidence for the presence of non semi-conservative mode of replication, referred to as repair replication, has been presented by Pettijohn and Hanawalt (1964). U.v. irradiated bacteria were allowed to grow in the presence of the more dense 5 - bromouracil (BrUra) instead of thymine. Caesium chloride density gradient centrifugation of the DNA isolated from these cells showed that BrUra was incorporated into very short segments along the DNA strands, at regions other than the normal replicating fork, since the incorporated BrUra can not be separated from DNA by either denaturing or shearing. Hanawalt and Pettijohn (1965) have shown that repair replication is operative in $uvr^+$ strains of E.Coli after exposure to u.v. light, but absent in the u.v. sensitive E.Coli B$_s$-1 mutant.

By measuring the post irradiation incorporation of exogenous [$^3$H] - thymidine into unreplicated DNA, containing heavy isotopes, Billen et al (1967) obtained data indicating the replacement by repair of about 50 nucleotides per dimer. However, the specific activity of the exogenously derived labelled thymidine might have been underestimated due to the dilution of label in the intracellular

DNA precursor pools.

The existence of non-conservative repair replication in mammalian cells, in response to u.v. irradiation or treatment with nitrogen mustard, has been demonstrated in many animal cells including Hela cells (Cleaver and Painter 1968) and in chinese hamster cells (Cleaver 1970a). Among mammalian cells, only rodent cells appear to have a reduced repair activity of this type (Painter and Cleaver 1970).

Xeroderma Pigmentosum (XP) is a human skin disease which follows an autosomal recessive pattern of inheritance (see Section 1.5). In each known case, exposure to sunlight causes a variety of phenotypic changes in skin cells, depending in severity upon the particular form of the disease. Fibroblasts derived from the skin of normal people can excise thymine dimers from their u.v. irradiated DNA, while cells from the skin of patients with XP do so at very much less than the normal rate (Setlow et al 1969).

Few enzymes which may participate in excision repair have been isolated from mammalian cells. Burt and Brent (1971) found a D Nase in Hela cells which specifically degrades the DNA of u.v. irradiated E.coli. Lindahl (1971) detected an enzyme activity in rabbit liver cell nuclei which can liberate dimers in oligonucleotide form from irradiated B. subtilis DNA.

Most of the eucaryotic repair activities which have been noted appear to correspond - albeit only approximately - to the uvr excision repair system of E.coli. The patches inserted appear to be shorter than those of bacterial systems. Edenberg

and Hanawalt (1972) found that they are confined to some 30 nucleotides in length.

The post u.v. irradiation, non semi-conservative DNA synthesis (DNA repair synthesis) can be referred to as "unscheduled" DNA synthesis or repair replication, depending on the technique used to demonstrate it. Evidence in favour of both terms describing the same biochemical phenomenon, has been presented (Painter and Cleaver 1969; Cleaver 1969a).

The incorporation of [$^3$H] - thymidine into cell nuclei which are not in the normal synthetic phase (S phase) following u.v. irradiation, is referred to as "unscheduled" DNA synthesis (Djordjevic and Tolmach 1967). This type of DNA synthesis can be demonstrated either by autoradiography or by measurement of total [$^3$H] - thymidine incorporation under certain conditions (see Methods Sections 2.1.5 & 2.1.6).

Another method to demonstrate repair replication is based on the incorporation of a heavy label such as 5 - bromodeoxyuridine (BrdUrd) into DNA (Pettijohn and Hanawalt 1964; Hanawalt and Cooper 1971). After exposure to u.v. light cells are allowed to grow in a medium containing [$^3$H] - BrdUrd an analogue of thymidine which is denser. The normal semi-conservative replication of DNA results in the formation of labelled "hybrid" density DNA, with a "light" parental and a "heavy" daughter strand. In repair replication on the other hand, only short regions of the DNA are excised and replaced by label-containing nucleotides and no density shift is expected. These two different modes of DNA replication can be resolved on a CsCl density gradient.

1.4.4 Post replication repair

Excision repair deficient mutants of E.coli can synthesize DNA after u.v. irradiation and the newly synthesized strands are of molecular weights inversely proportional to the u.v. dose (Rupp and Howard-Flanders 1968; Smith 1969). If these cells are incubated long enough after irradiation these defects in the newly synthesized DNA disappear. The disappearance of these defects has been attributed to a post replication repair process, also referred to as "recombinational repair".

Mutations in the recombination system of E.coli, which comprises three loci - rec B and rec C which are closely linked and rec A which maps at a different location - cause an increased sensitivity to both u.v. and x-irradiation (Howard-Flanders et al 1966). Mutants of E.coli which are defective in recombination fall into two classes. The "reckless" mutants in rec A suffer a drastic reduction in recombination and are very sensitive to u.v. irradiation, which causes them to degrade an abnormally large amount of their geromes. The "cautious" mutants in rec B or rec C have reduced but detectable recombination, increased sensitivity to u.v. light, and a smaller extent of breakdown of DNA after irradiation which is comparable in amount to that of the wild type. This suggests that the product of the rec B and rec C genes may be a nuclease which is responsible for much of the breakdown of DNA after u.v. irradiation, whereas the product of the rec A gene in some way limits the process. This idea is supported by the behaviour of the double mutants constructed by Barbour and Clark (1970); rec A$^-$ rec B$^-$ or rec A$^-$ rec C$^-$ mutant

have lower levels of breakdown after u.v. irradiation than those exhibited by rec A⁻ mutants or wild type cells.

A crucial difference between the uvr and rec systems is that excision-repair acts on dimers themselves in the parental strands of DNA, whereas recombination repair acts on the gaps left in daughter strands which are produced by replication of unexcised dimers.

DNA molecules that have been damaged in both strands at the same point cannot be repaired by an excision mechanism, since there is no intact complementary strand to serve as a template for repair synthesis (Kelly et al 1969; Cole 1971). DNA molecules that have suffered two-strand damage can, however, be repaired through recombination with homologous molecules. The base sequences needed to accurately reconstruct the molecule at sites of two-strand damage are available in the homolog and can be brought into use through genetic exchange.

Fig. 6 shows a schematic representation of the recombination repair process suggested by Howard-Flanders (1973). It has been shown by Rupp and Howard-Flanders (1968) that the DNA synthesized at early times after u.v. irradiation is of molecular weight approximately equal to that of the DNA chains between the u.v. induced pyrimidine dimers. This result suggests that the newly synthesized DNA chains terminate at pyrimidine dimers in the template DNA, and that new chains are started at initiation sites further along the template strands. The resulting gaps opposite pyrimidine dimers can be 500 - 1000 nucleotides in length (Iyer and Rupp 1971). The post replication gap filling in u.v. irradiated

Fig. 6 Schematic representation of the post-replication repair process proposed by Howard-Flanders (1973).

a) a DNA duplex containing a thymine dimer

b) the duplex containing the dimer is replicated. One newly synthesised strand is terminated at the dimer and a new strand is started at an initiation point beyond, leaving a post-replication gap. The thymine dimer is not subject to the action of excision enzymes while in a single-strand region

c) the strands at the post-replication gap initiate a genetic exchange with an intact portion of the homologous sister duplex. One strand in the sister duplex is cut in response to pairing, and is joined in the configuration shown.

d) the post-replication gap opposite the dimer is filled with the correct base-pair sequence by a change in the complementary hydrogen bonding between strands, as shown.

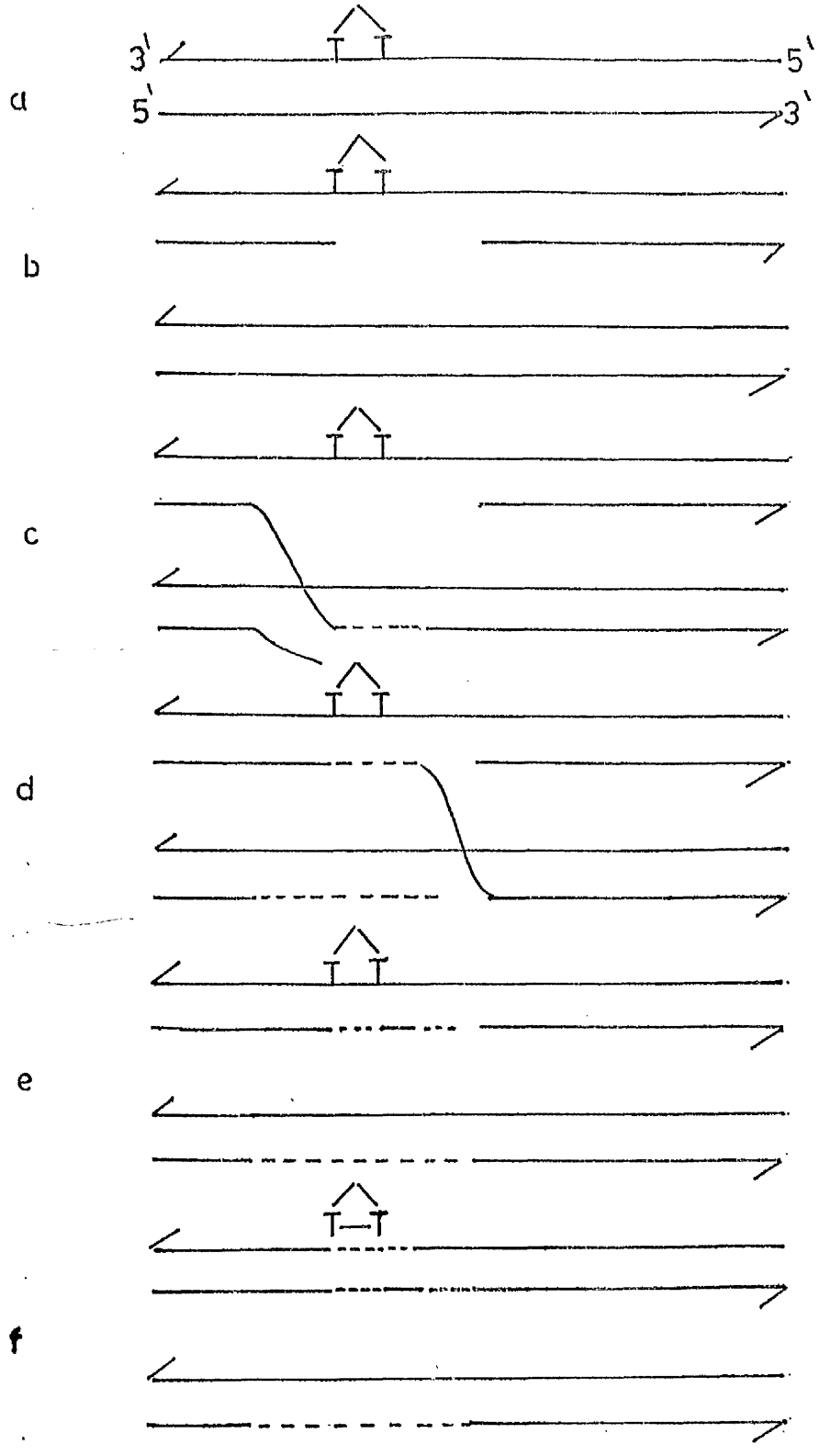
e) the sister exchange is completed by the strand linking the two duplexes being cut, so that both duplexes can be repaired.

f) after the post-replication gap is filled, the thymine dimer will be excised and the strand finally repaired.

———— Original strand

– – – – – – Repaired strand

T—T thymine dimer


a
3'
5'
5'
3'
b
c
d
e
f

bacteria appears to involve sister strand exchanges, and these can be detected by means of heavy isotope density labels. When cells are grown so that the DNA is a density hybrid with one heavy and one light strand, it is normally possible to extract the DNA and to separate heavy and light strands cleanly by denaturing the DNA and centrifuging it in a CsCl equilibrium density gradient (Meselson and Stahl 1958). If, however, the cells are exposed to low doses of u.v. light before the synthesis of the light strands commences, single strands of intermediate density are obtained. These occur at a yield of one intermediate density molecule for every one to two pyrimidine dimers in the replicated DNA and are presumably formed by sister-strand exchanges. The conclusion derived from these experiments is that dimers and post-replication gaps cause genetic exchanges with a high efficiency (Rupp et al 1971).

Another type of two-strand damage, where post-replication repair may be a candidate is inter-strand cross-links. Bifunctional alkylating agents, such as nitrogen and sulphur mustards and their derivatives, form diguaninyl products in DNA, of which one quarter or more are inter-strand cross-links (Lawley et al 1969). Cross-links are also produced by Cis-platinum (II) diaminodichloride (Roberts and Pascoe 1971).

In bacteria and animal cells, cross linked DNA is acted upon by excision enzymes, releasing the cross-links so that strands can separate when denatured (Kohn et al 1965; Lawley and Brookes 1965; Cole 1973). It is likely that excision enzymes make two cuts in one strand at each cross-link, producing a gap in one strand while

the half-excised cross-link remains attached to the other (Cole 1973; Howard-Flanders and Lin 1973). A half-excised cross-link is analogous to a pyrimidine dimer and post-replication gap, in that both structures involve a single-strand gap that can not be immediately repaired because of the damage in the single-strand region. These two structures might cause exchanges through a post replication repair type of mechanism.

Although a process of post-replication repair similar to that in bacteria appears to exist in mammalian cells, some controversial points have arisen concerning the mechanism of this process in mammalian cells. Thus, in u.v. irradiated chinese hamster cells, it was found that the percentage of thymine-containing dimers in replicated and unreplicated DNA is the same (Heyn et al 1974). In mouse fibroblast cells, in which excision occurs very slowly, the synthesis of high-molecular weight DNA after u.v. irradiation can be detected before the end of the first doubling time after irradiation. This implies that synthesis of high molecular weight DNA occurs before there is time for the post-replication gap filling to be followed by the next synthetic period (Lehmann 1972). This suggests that repaired templates become available for synthesis more rapidly than can be accounted for on the basis of the model (Fig. 6 ), derived from bacteria in which, after exposure to u.v. light, replication is followed by recombination. High molecular-weight DNA should then be synthesized only after the next synthetic period. The situation in animal cells is also complicated by the need to avoid extensive crossing-over leading

to loss of heterozygosis during embryonic development. The exploration of induced recombination and conversion in somatic cells is hampered by the lack of genetic markers that can be arranged so that recombinants and convertants can be selected for.

The deployment of the post-replication process in the repair of non dividing cells such as lymphocytes, is difficult to envisage, since in most biological systems genetic recombination is associated with DNA replication. However, certain types of bacteriophage are known where recombinants can be formed by cutting and joining pre-existing DNA molecules without the necessity for replication, other than that which may be needed for repair synthesis (Meselson 1964; McMilin and Russo 1972).

All mammalian cells studied so far are able to carry out post-replication repair, i.e. they all eventually produce high molecular weight daughter-strand DNA after u.v. irradiation. However, fibroblasts derived from patients suffering from the disease XP "variant" strain (section 1.5), have abnormally low rate of post-replication repair (Lehmann et al 1975), which is inhibited even further in the presence of Caffeine.

## 1.5 Xeroderma pigmentosum

Xeroderma pigmentosum, first identified a century ago (Hebra and Kapusi 1874), is a rare genetic disorder which is generally inherited as an autosomal recessive trait (Reed et al 1969), though a partial incomplete sex-linked recessive pattern has also been reported (Anderson and Begg 1950; El-Hefnawi et al 1962). The disease effects both sexes equally, occurs in all races and has been described in almost all parts of the world.

The skin of the homozygotes is normal at birth and changes usually appear between 6 months and 3 years of age though occasionally evidence of the disease does not occur until adult life. This delayed appearance may be due in part to opportunities for sun-exposure and the degree of expression of the genetic defect.

Essentially all the cutaneous changes induced in XP are produced by sun exposure and thus the lesions occur primarily on the areas receiving the greatest amount of solar radiation (Plate 1).

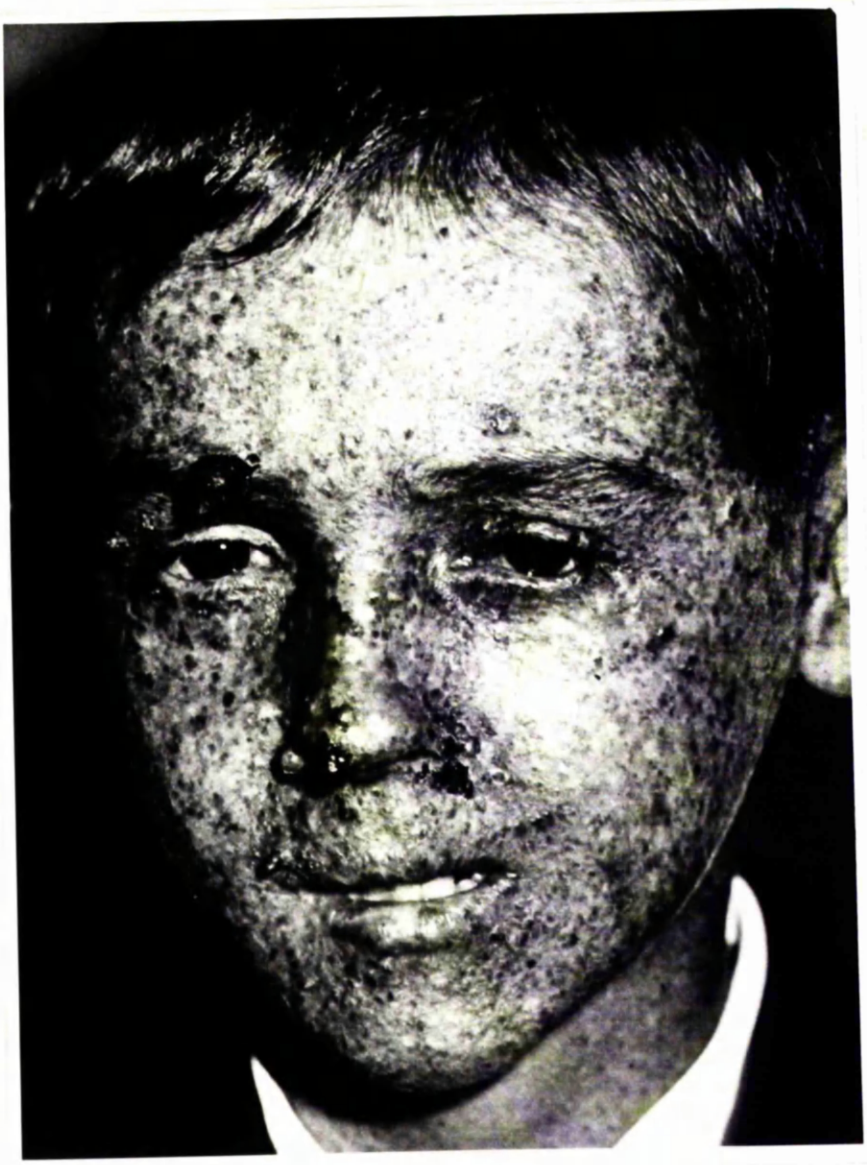
These lesions develop into tumours, and the most common tumours which frequently occur early in the course of the disease are keratoacanthomas (Reed et al 1969). Malignancies of epidermal origin (basal cell epitheliomas and squamous cell carcinomas) are also common. However, all varieties of cutaneous cancer may occur including malignant melanomas (El-Hefnawi et al 1962; Lynch et al 1967).

There are two clinical forms; both show the skin symptoms but the rare form identified by De Sanctis and Cacchione (1932) is usually more severe and is associated with neurological complications (Reed et al 1965).

The wavelengths responsible for the changes in XP appear to fall

Plate 1 Xeroderma pigmentosum in a 9 year old boy, showing widespread keratoses with neoplastic change most evident above the right eye and on nose.

Courtesy of Dr. R.M. Mackie, Department of Dermatology, Western Infirmary, Glasgow.



in the sunburn spectrum 290 - 320 nm (Lynch 1934). The minimal erythema dose of these rays is generally normal (Rothman 1923). However, a number of abnormal responses have been reported. These include a delay in the appearance and/or a reduction in the size of the peak of the erythema response (Martenstein 1924), persistence of the erythema for abnormally long time (Cripps et al 1971) and chronic sun damage (Nurnberger 1968).

Gartler (1964) reported that fibroblasts cultured from the skin of patients with XP were abnormally sensitive to u.v. irradiation. Cleaver (1968) demonstrated that repair of u.v. induced pyrimidine dimers by the dark-repair system was defective in XP fibroblasts. These findings were confirmed subsequently by a number of studies utilizing various techniques ( etlow et al 1969; Cleaver 1970b; Bootsma et al 1970; Regan et al 1971). The repair deficiency in XP patients is not confined to fibroblast cultures, but is also found in non dividing cells such as lymphocytes (Burk et al 1971a). However, it has been shown by Robbins and Kraemer (1972a, 1972b) that while lymphocytes from patients with XP show a lower level of repair compared to normal lymphocytes immediately after u.v. irradiation, they continue to repair beyond the time required for normal lymphocytes to complete their repair. The incorporation of [$^{3}$H] thymidine (a measure of repair) into irradiated XP lymphocytes ceases only when they have incorporated as much thymidine as the normal irradiated lymphocytes.

Until recently all of the cells from patients with characteristic clinical cutaneous changes of XP have demonstrated the dark-repair enzyme defect. Burk et al (1971a, 1971b) reported normal post-u.v. repair in lymphocytes and in fibroblasts of a patient with severe

classical XP. Subsequently, Cleaver (1972) noted normal repair replication in the fibroblasts of two other patients with apparent characteristic XP. These cells were not abnormally sensitive to the killing effect of u.v. light, indicating that they did not have a defect in any aspect of the excision or any other repair system. This form of XP is referred to as the "XP variant". Although the excision repair system appears to be normal (Robbins et al 1974), low levels of photoreactivating enzyme (Sutherland and Oliver 1975) and a defective post replication repair (Lehmann et al 1975) in XP variants have been reported. In addition, it has been demonstrated by Maher et al (1976) that the frequency of u.v. light induced mutations is higher in XP variant cells than in normal human cells. This finding supports the somatic cell mutation hypothesis on cancer first put forward by Boveri (1929).

Apart from the XP variant, there now exist five distinct complementation groups in XP patients whose cells exhibit defective DNA repair ranging from (2 - 50%) that of normal cells (Kleijer et al 1973; de Weerd-Kastelein et al 1973; Bootsma et al 1975). Moreover, the ability of XP cells to perform repair replication (section 1.4.2) seems to vary with the severity of the disease; for Bootsma et al (1970) showed that there is a correlation between the overall ability of the cells to perform repair replication of u.v. irradiated DNA and the clinical severity of the condition.

An early attempt to study the biochemical basis for the repair deficiency in XP cells led Cleaver (1969b) to suggest that an initial stage in the excision repair is defective. This was inferred after it was found that XP fibroblasts can repair x-ray damage (which causes chain breakage, Freifelder 1968) but not base damage (without chain

breakage) caused by u.v. light (Setlow 1966). This is because the repair of damage to DNA bases requires enzymatic scission of the polynucleotide chain (Setlow et al 1969), excision of damage and repair replication (Hanwalt 1968), while the repair of strand breaks does not require the initial enzymatic chain scission, but may involve the later stages (Painter and Cleaver 1967). Setlow et al (1969) demonstrated that the excision of u.v. light induced pyrimidine dimers from DNA of XP fibroblasts is markedly reduced. However, when extracts of XP cells examined for dimer excising activity on previously incised DNA with a dimer-specific endonuclease, purified from bacterophage $T_4$ - infected E.coli (Friedberg and King 1971), no significant difference was found in the specific activity of excision nuclease in any of the XP classes, compared with normal cells (Cook et al 1975).

Since extracts of cells from normal individuals can carry out DNA repair on chromatin from u.v. irradiated XP cells, while XP extracts cannot (Mortelmans et al 1976), it was concluded that repair must be considered at the level of chromosome structure as well.

Host cell reactivation experiments conducted with XP fibroblasts showed that these cells have little or no ability to reactivate u.v. damaged herpes or $SV_{40}$ viruses (Rabson et al 1969; Aaronson and Lytle 1970). Studies on DNA repair synthesis in $SV_{40}$ transformed XP cell strains indicated that the original defect can not be rectified by the integration of $SV_{40}$ genetic material (Bootsma et al 1970; Cleaver 1970; Parrington et al 1971).

Since XP cells do not appear to lack the repair activity completely, the different levels of repair activity found, may be due to different mutations in the same gene. This can give rise to

different degrees of impairment of the endonuclease responsible for lesion recognition (Cleaver 1970b). However, there is strong evidence showing that two different genes are involved in the basic defect of de Sanctis-Cacchione syndrome and the classical form of XP (Der Kaloustian et al 1974). This conclusion was reached after observing normal level of repair replication in heterokaryons containing nuclei from cells of both the de Sanctis-Cacchione syndrome and the classical form of XP (De Weerd-Kastelein et al 1972).

In addition to the different complementation groups of XP, there exist another disorder in which light produces skin alterations resembling XP, observed by Jung (1970). This condition has been referred to as "pigmented xerodermoid" and classified as a condition between extensive solar keratosis and a mild form of XP. Although epidermal cells from patients with pigmented xerodermoid have a normal level of repair replication after exposure to u.v. radiation, even relatively low doses of u.v. light result in almost total depression of the semi-conservative DNA synthesis in the pigmented xerodermoid basal cells.

## 1.6 Actinic Keratosis

Actinic keratosis (AK) is a premalignant lesion of the epidermis. Extensive exposure of the skin to sunlight is generally considered as an important factor in the etiology of the disease (Pinkus 1971).

The lesions appear as rough pigmented raised areas on the exposed parts of the skin (Plate 2). They are seen at an earlier age in patients who have an outdoor occupation (e.g. farmers, seamen) and most strikingly in fair skinned people who emigrated to the southern

Plate 2 Actinic keratosis in a 58 year old man with keratoses on dorsum of both hands.

Courtesy of Dr. R.M. Mackie, Department of Dermatology, Western Infirmary, Glasgow.

hemisphere. The disease effects both sexes and if left untreated may slowly progress to squamous cell carcinoma in 12 to 13% of the affected individuals (Graham and Helwig 1972). Hereditary factors or some other common predisposition to the development of cancer is suggested by the occurrence of skin tumours and internal malignant neoplasms in one or more members of the families in half the patients with AK (Graham and Helwig 1972).

The demonstration by Cleaver (1968) that fibroblasts from patients suffering from the sun-sensitive disease XP (see Section 1.5) are defective in DNA repair after exposure to u.v. light, opened the possibility that repair deficiency may be involved more generally in carcinogenesis. This suggestion was supported by the finding, that a correlation exist between the overall ability of XP cells to perform repair replication of u.v. irradiated DNA and the severity of the disease (Bootsma et al 1970). The argument is further strengthened by the demonstration that neoplastic transformation can result from the presence of thymine dimers in cells irradiated with u.v. light (Hart and Setlow 1975).

Although the progress of AK and that of XP are different, similarities exist between the two diseases:

(1) Both XP and AK are sun-sensitive conditions which give rise to skin lesion

(2) These lesions develop into skin tumours, though with differing rates of incidence. There is a high incidence at an early age in the case of XP and a lower incidence usually among elderly people (after the age of 50 years) in the case of AK.

While XP is genetically determined, the genetic basis of AK (if any) is not yet established.

In order to decide whether or not a deficiency in repair is the common biochemical basis of both diseases, a study of DNA repair in cells from AK patients was undertaken and is the subject of this thesis.

A less extensive study of DNA repair in "melanoma" and "basal cell carcinoma" for sunlight was included as it is recognized that sunlight also plays a role in the aetiology of these neoplastic conditions (Elliott and Welton 1946; McGovern 1952; Belisario 1954; Lancaster 1955) and also because a small percentage of AK patients develop such tumours (Graham and Helwig 1972).

Unscheduled DNA synthesis (section 1.4.2) can be studied in non-dividing cells such as lymphocytes (Evans and Norman 1968; Lieberman et al 1971). When studying repair the lymphocytes have a distinct advantage over the fibroblasts for the following reasons:-

(1) Lymphocytes are easily obtained from freshly drawn blood and repair can be assayed within hours (C.f. fibroblasts cultures derived from skin biopsies which take months to establish).

(2) Several million lymphocytes are used in each determination and these form a representative population whereas the few cells which grow from a skin biopsy are highly selected and may not be representative samples.

(3) Lymphocytes are generally not dividing ( $< 1\%$ dividing cells) and do not synthesize DNA without prior stimulation (e.g. by PHA). After PHA stimulation about 80% of the cells synthesize DNA which is a measure of the viability of the population. Hence in the absence of DNA stimulation the $[^3H]$ - dT incorporation due to repair after u.v. irradiation forms a high proportion of the total incorporation (C.f. incorporation

in u.v. irradiated fibroblasts where DNA replication completely masks repair synthesis).

The study of DNA repair in cells derived from AK patients may provide further understanding of the relationship between the state of DNA repair and carcinogenesis.

## 2. METHODS AND MATERIALS

## 2.1 Methods

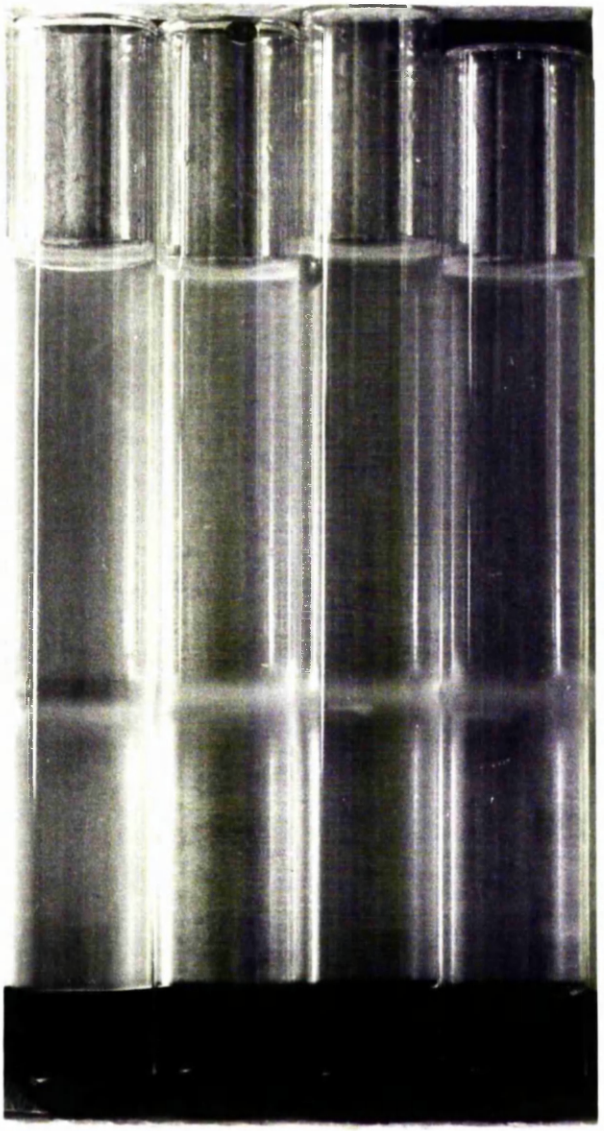
### 2.1.1 Isolation of lymphocytes

Lymphocytes were isolated by a modification of the method described by Boyum (1968). The isolation procedure depends basically on two physical properties, cell size and cell density. Heparinised blood (200 units heparin/10 ml of blood) was diluted 1 : 1 with isotonic saline (see materials section) to increase the relative osmolarity of the suspending medium. The relative osmolarity of a solution is defined as the osmolar concentration of that solution relative to the osmolar concentration of blood plasma. Increasing the osmolarity of the suspending medium causes the cells to lose water, hence their density increases. This results in a more rapid sedimentation rate.

The separation fluid (see materials section) contained Ficoll 400 (8% w/v), which selectively aggregates erythrocytes (Richer 1963), and sodium metrizoate (32.8% w/v), which because of its high density, prevents the complete sedimentation of lymphocytes (Boyum 1964).

The diluted blood (in 4.0 ml lots) was layered gently on to 3.0 ml lots of separation fluid in test tubes (11 x 100 mm) and allowed to stand for 10 mins. The tubes were then centrifuged at 400 g for 30 min. at 20°C. The erythrocytes and granulocytes aggregate and hence sediment through the separation fluid and collect as a firm pellet at the bottom of tube, while the lymphocytes and platelets separated as bands near the interface (see Plate 3). The lymphocyte bands were aspirated with a pasteur pipette and pooled. Two drops of foetal calf serum were added and the suspension divided into 2.5 ml aliquots in graduated 15 ml centrifuge tubes. Each aliquot

Plate 3 The separation of white blood cells from erythrocytes on a Ficoll sodium metrizoate gradient. Erythrocytes collect at the bottom of the tube while white blood cells form a layer at the interface, from which lymphocytes can be isolated (see methods section 2.1.1)



was made up to 5.0 ml with phosphate buffered saline (PBS) pH 7.2 (see materials section 2.2.4) and centrifuged at 150 *g* for 30 min at 20°C. The supernatants which contained most of the platelets were discarded. The pelleted lymphocytes were resuspended in 1 - 2 ml PBS per tube, pooled and centrifuged at 200 *g* for a further 10 min. The supernatant was again discarded and the pellet, which contained 98 - 99% lymphocytes was resuspended in 2.0 ml PBS and counted.

The yield of lymphocytes obtained by this method ranged between 1.0 and 1.5 million cells/ml of blood, which compares well with that (1 million cells/ml of blood) obtained by Cooper & Rubin (1965).

The viability of the lymphocytes prepared in this way from both AK patients and normal individuals was tested by assaying their response to phytohaemagglutinin (PHA) stimulation using $[^3H]$ - dT incorporation (see methods section 2.1.7). The cells from both types of patients show similar increases in $[^3H]$ - dT incorporation (Fig. 23A), and autoradiographic examination (see Plate 6) showed that in both cases nearly 80% of cells synthesize DNA after 48 h incubation with PHA (Fig. 23B). This value is very close to those reported by Cooper (1968) and Frey-Wettstein *et al* (1969).

### 2.1.2 Lymphocyte culture

Lymphocytes were derived from the peripheral blood of patients with AK and age-matched normal individuals, and occasionally from patients with other sun-sensitive conditions. The blood was supplied by the Department of Dermatology, Western Infirmary, Glasgow.

Cells were counted in an "Improved Neubauer" haemocytometer. Lymphocytes were distinguished from red blood cells by their morphology and by counting the cells before and after treatment with 1% acetic acid which lyses the red cells but not lymphocytes.

Lymphocytes ($3.0 \times 10^6$) were cultured in 50 mm diameter petri dishes (Nucleon) containing 4.0 ml Eagle's medium (see materials section) supplemented with 15% foetal calf serum ($FC_{15}$).

Cell cultures were maintained at $37^{\circ}C$ in a humidified incubator flushed with an atmosphere of 5% $CO_2$ in air.

2.1.3 Irradiation of cells with ultra violet light

Lymphocytes suspended in 1.0 ml PBS pH 7.2 ($3.0 \times 10^6$ cells/ml), were placed in 50 mm diameter plastic dishes, shaken gently to ensure even distribution of cells in the dish and then exposed to u.v. light. The incident dose of u.v. radiation source was adjusted to 0.5 $J.m^{-2}.sec.^{-1}$ The energy emission was determined with a potassium ferrioxalate actinometer (methods section 2.1.4). Unirradiated control suspensions were prepared and treated similarly except for irradiation. 3.0 ml $FC_{15}$ was added to all the dishes and the cultures transferred to the incubator.

2.1.4 Calibration of the ultra violet radiation source

The intensity of u.v. light, emitted from a germicidal lamp which gave light predominantly at a wavelength of 254 nm, was determined with a potassium ferrioxalate chemical actinometer (Hatchard and Parker 1956).

The method depends on the photolysis of an acidified solution of the ferric iron complex, potassium ferrioxalate $K_3\ Fe(C_2O_4)_3\ 3H_2O$ which results in the liberation of ferrous iron. The amount of ferrous iron formed depends on the u.v. radiation dose applied and can be determined by a colorimetric method. Potassium ferrioxalate is chosen for the preparation of actinometer solution, because it

forms a convenient quantitative standard, is readily prepared by mixing 3 volumes of 1.5 M A.R. potassium oxalate with 1 volume of 1.5 M A.R. ferric chloride with vigorous stirring, has an accurately known and reproducible composition and can be stored indefinitely.

Estimation of ferrous Iron

The actinometric procedure depends on the estimation of ferrous iron. Various volumes (0, 0.5, 1.0, .... 4.5, 5.0) 4 x $10^{-4}$ M ferrous sulphate, freshly diluted with 0.1 N $H_2SO_4$ from a standard solution of 0.1 M ferrous sulphate in 0.1 N $H_2SO_4$, were added to a series of tubes. The volumes were made up to 10 ml with 0.1 N $H_2SO_4$. To each tube 2.0 ml 1, 10- phenanthroline solution (0.1% w/v) and 5.0 ml actinometer buffer solution (see materials section 2.2.4) were added. The solutions were made up to 20 ml with water, mixed thoroughly, allowed to stand for 30 min. and their extinctions at 510 nm measured. The calibration curve for the estimation of ferrous iron is shown in Fig. 7

General procedure for actinometry

18.0 ml of photolyte (6 x $10^{-3}$ M potassium ferrioxalate in 0.1 N $H_2SO_4$) were irradiated in a petri dish (50 mm in diameter) for 15 min at a distance of 0.125 m from the u.v. source. Aliquots of the irradiated photolyte (see Table 1) were pipetted into a series of tubes and 2.0 ml 1, 10 - phenanthroline solution together with a volume of actinometer buffer solution equal to half that of photolyte was added and the solutions mixed.

Calibration of the ultra violet radiation source

| Photolyte (ml) | $E_{510}$ | | $\Delta E$ | Moles x $10^{-6}$ $FeSO_4$ | |
|---|---|---|---|---|---|
| | Irradiated Photolyte | Unirradiated Photolyte | | Per Aliquot | Per 18.0 ml |
| 0.5 | 0.138 | 0.062 | 0.066 | 0.12 | 4.32 |
| 1.0 | 0.200 | 0.065 | 0.135 | 0.24 | 4.32 |
| 1.5 | 0.264 | 0.068 | 0.196 | 0.34 | 4.08 |
| 2.0 | 0.334 | 0.070 | 0.264 | 0.44 | 3.96 |
| 2.5 | 0.400 | 0.074 | 0.326 | 0.56 | 4.03 |
| 3.0 | 0.482 | 0.078 | 0.404 | 0.68 | 4.08 |

Table 1

18 ml of 0.006 M potassium ferrioxalate in 0.1 N $H_2SO_4$ in a 50 mm diameter petri dish was irradiated for 15 min at 0.125 m below the u.v. source. Aliquots ranging between 0.5 - 3.0 ml of photolyte were used for the determination of the released $Fe^{2+}$ (expressed in moles $FeSO_4$ x $10^{-6}$). $\Delta E$ is the difference in extinction measured at 510 nm before and after irradiation.

The solutions were made up to 20 ml with water, left standing for 30 min. in the dark and their extinctions measured at 510 nm. The procedure was repeated with the same volumes of unirradiated potassium ferrioxalate solution. The difference in extinction ($\Delta$ E) between the irradiated and unirradiated solutions was converted into moles ferrous salt formed using the calibration graph (Fig. 7).

The quantity of ferrous salt formed in the total volume of irradiated solution was converted to a radiation dose using the quantum yield given by Hatchard and Parker (1956) and the relation:-

$$\text{Quantum Yield} = \frac{\text{Number of ferrous ions formed}}{\text{Number of quanta absorbed}}$$

The quantum yield depends on the wavelength of radiation and was estimated by Hatchard and Parker (1956) as 1.25 for 254 nm. The number of quanta absorbed was converted to Joules using Einstein's equation:-

$$\text{Energy of 1 quantum} = \frac{h \times C}{\lambda(\text{in nm})}$$

Where h is Planck's constant = $6.62 \times 10^{-27}$ erg : sec

C is the velocity of light = $2.9977 \times 10^{10}$ cm . $sec^{-1}$

$\lambda$ is the wavelength in (nm) = $254 \times 10^{-7}$ cm

Substituting these values

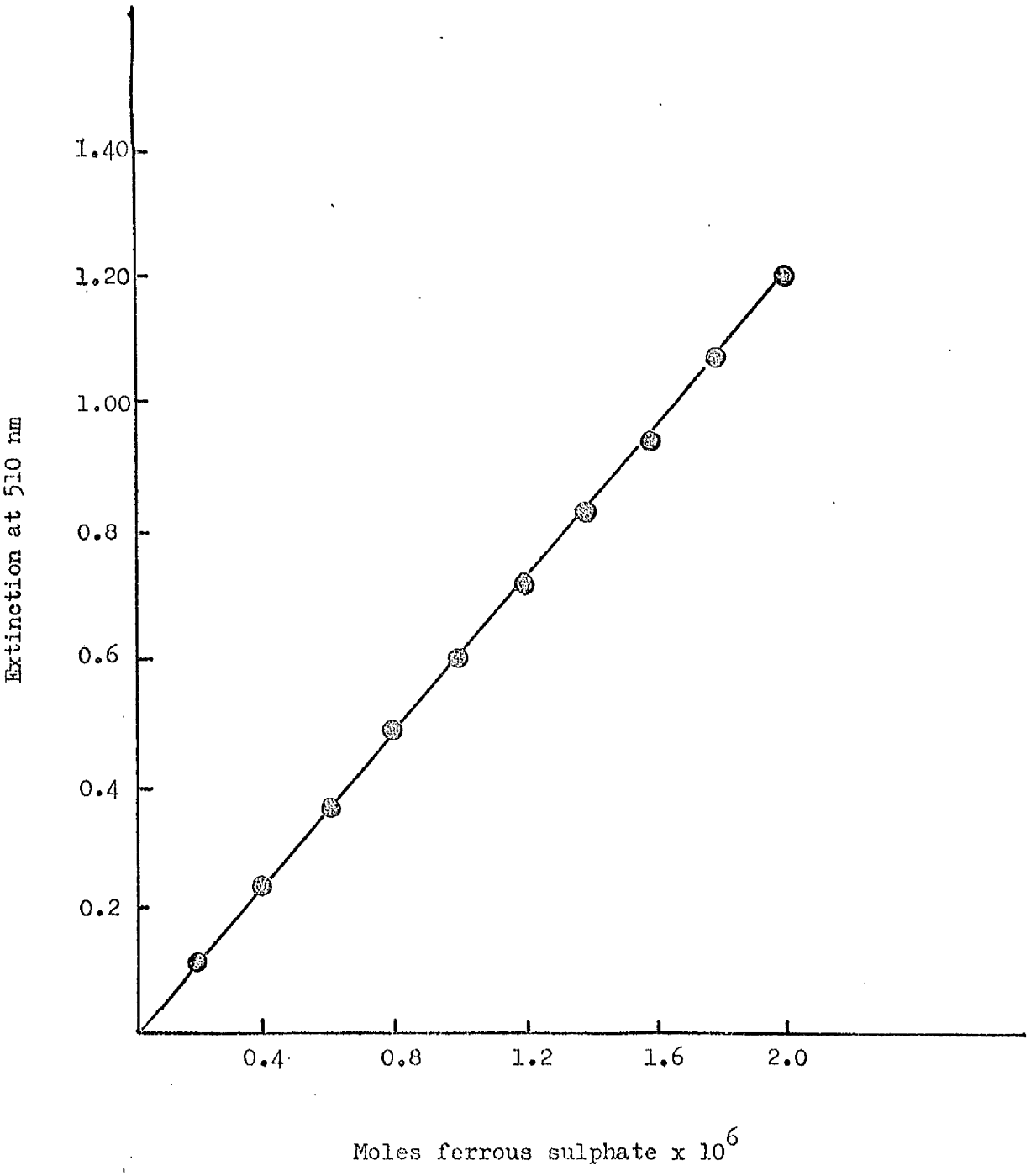
$$\text{thus 1 Quantum} = \frac{6.625 \times 10^{-27} \times 2.9977 \times 10^{10}}{254 \times 10^{-7}}$$

$$= 7.8 \times 10^{-10} \text{ ergs}$$

$$= 7.8 \times 10^{-7} \text{ Joules} \quad (1 \text{ Joule} = 10^{7} \text{ erg})$$

Fig. 7 Calibration curve for $FeSO_4$ determination

Two samples of $FeSO_4$, in 10 ml 0.1 N $H_2SO_4$, 2.0 ml of 1, 10 - phenanthroline solution (0.1% w/v) and 5.0 ml actinometer buffer solution (see materials section 2.2.4 ) were added and the volumes adjusted to 20 ml with water. After 30 min. the extinction of the solutions was measured at 510 nm and the quantities of $FeSO_4$ in (moles per assay) were plotted against the corresponding extinction measurements.


Extinction at 510 nm
1.40
1.20
1.00
0.8
0.6
0.4
0.2
0.4
0.8
1.2
1.6
2.0
Moles ferrous sulphate x $10^6$

The mean value for the total number of moles of $FeSO_4$ equivalent to the amount of ferrous ions liberated by the irradiation of 18.0 ml of photolyte was calculated from Table 1 to be $4.13 \times 10^{-6}$ moles $FeSO_4$.

This corresponds to 1.56 joules/dish/15min

The surface area of the petri dish (0.05 m diameter) = $1.963 \times 10^{-3}$ $m^2$

∴ When the u.v. source was situated at a distance of 0.125 m above the irradiated area, the energy emission under these conditions was calculated to be 0.88 $J.m^{-2}.sec^{-1}$

The height of the lamp was adjusted to give the desired energy emission, using the following relationship:-

$$\frac{E_1}{E_2} = \left(\frac{h_1}{h_2}\right)^2$$

Where $E$ = energy of emission of the u.v. source in $J.m^{-2}.sec^{-1}$

$h$ = height of the u.v. source above the irradiated area in m

When the lamp was further calibrated with a new height of 0.165 m from the irradiated surface, the energy of emission was found to be 0.505 $J.m^{-2}.sec^{-1}$. This value is almost identical to the one obtained from the relation

$$E_1/E_2 = \left(\frac{h_1}{h_2}\right)^2$$

i.e. $$\frac{0.88}{E_2} = \left(\frac{0.125}{0.165}\right)^2$$

∴ $$E_2 = 0.500\ J.m^{-2}.sec^{-1}$$

2.1.5 Estimation of DNA repair by autoradiography

Lymphocyte cultures were labelled by adding [$^3$H] - dT (5.0 $\mu$Ci/ml, 18.5 Ci/mmole) immediately after irradiation and incubating the cultures at 37°C. for 4 h. The labelled medium was then transferred into a marked graduated tube. The cells sticking to the bottom of the dish were scraped with a rubber policeman into 2.0 ml PBS containing a few drops of serum, and combined with the previously removed medium. The suspension was centrifuged at 400 g for 10 min at 4°C and the supernatant discarded. The pellet was washed three times by suspending the cells in 5.0 ml PBS, centrifuging as before and discarding the washings. Slowly, while mixing with a mechanical shaker, 0.5 ml Carnoy's fixative (2.2.4) was added to the pellet dropwise to fix the labelled DNA and preserve the cellular morphology (Craddock et al 1964). About 0.05 ml of a fixed suspension was smeared on a dry clean microscope slide and left to dry. The slides were then washed twice with ice-cold 5% TCA, once with ice-cold distilled water and finally rinsed once with methylated spirit at room temperature. In the darkroom fitted with a Wratten 1A safe light, slides were dipped in Ilford L4 emulsion, allowed to dry for a few minutes and stored in a dark, airtight box with silica gel as a dessicant. After eight days exposure, the autoradiographs were developed with Kodak D19b for 5 min at 18°C, fixed in Amfix for 4 min at room temperature and stained for ½ min at room temperature with Giemsa, freshly diluted 1 : 20 (v/v) in water.

Grains were counted over the lightly labelled nuclei (always more than 99% of the cells), see figures 11, 12 & 13,

and the arithmetic mean and the standard deviation were calculated in each case. The mean background grain count was estimated by counting the grains over cell-free regions of the same area as that of the cell nucleus and substracted from the mean value in each experiment.

2.1.6 Measurement of DNA repair by [$^3$H] - dT incorporation into the acid-insoluble material of u.v. irradiated lymphocytes in the presence of hydroxyurea (OH-urea)

The method is a modification of the technique described by Evans and Norman (1968). Lymphocyte cultures were labelled by adding [$^3$H] - dT (5.0 $\mu$Ci/ml, 18.5 Ci/mmole) immediately after irradiation. OH-urea (final concentration $1.5 \times 10^{-3}$ M) was added at the same time as the label to inhibit DNA replication ( Weaver 1964a) in the few (<0.1%) dividing cells (Bond et al 1958; Frey-Wettstein et al 1969). After incubating at 37°C for 4 h, the cells were harvested and washed by centrifugation as described in methods section 2.1.5. They were fixed in 0.5 ml Carnoy's fixative as before, which allowed the simultaneous analysis of repair by autoradiography. A known volume (usually 0.2 ml) of the fixed cell suspension was filtered through a Whatman glass fibre 2.5 cm filter. The filter was washed 4 times with 2.0 ml ice-cold 5% TCA (w/v), rinsed with 2.0 ml methylated spirit, transferred to a scintillation vial and allowed to dry for at least 2 h in a 60°C oven. The precipitate was dissolved in 0.5 ml hyamine hydroxide (10 min at 60°C) to increase the counting efficiency and allow the efficiency to be estimated by the channels ratio method. 5.0 ml toluene-based scintillation fluid (materials section 2.2.4) was added to the

cooled scintillation vials and counted in a Nuclear Chicago IsoCap/300 liquid scintillation spectrometer.

The u.v. stimulated [$^3$H] - dT incorporation (DNA repair) was calculated by substracting the incorporation (d p m) into unirradiated cells from the incorporation into irradiated cells.

2.1.7 Estimation of DNA repair by [$^3$H] - dT incorporation into acid-insoluble material of lymphocytes stimulated with phytohaemoagglutinin (PHA) and subsequently irradiated with u.v. light

35 mm petri dishes containing 1.0 x $10^6$ lymphocytes in 2.0 ml RPMI15 medium containing 50 units PHA (see materials section 2.2.1) were incubated at 37°C for a minimum of 40 h. The cells were then separated by centrifugation (400 g for 10 min) at 20°C. The supernatant media were removed from the pellets and kept separately. Pellets were resuspended in 1.0 ml phosphate buffered saline (pH 7.2) and placed in 50 mm petri dishes for irradiation. Half the cultures were irradiated, as described in section 2.1.3 at 20 J.m$^{-2}$ and the original media supplemented with 1.0 ml fresh RPMI15 were added back. The cultures were again incubated at 37°C. Irradiated cultures and the same number of unirradiated control cultures were labelled with [$^3$H] - dT (0.5 $\mu$Ci/ml, 18.5 Ci/mmole) for 1.0 h periods, 0-1 h, 1-2 h, 2-3 h, 3-4 h after irradiation. After the 1 h labelling period, the cultures were harvested, washed and fixed in Carnoy's fixative as described in section 2.1.6. Samples were further processed for autoradiography and or the estimation of total incorporation (see methods sections 2.1.5 & 2.1.6).

2.1.8 CsCl equilibrium density gradient centrifugation

Lymphocyte cultures ($3 \times 10^6$ cells in 5.0 cm dishes) were cultured and irradiated with u.v. light (20 $J.m^{-2}$) as described in methods section 2.1.3 and then incubated at 37°C for 4 h in 4.0 ml media containing 6 [$^3$H] 5 - bromo - 2' deoxyuridine ([$^3$H] - BrdUrd, 10 $\mu$Ci/ml 2.2 Ci/mmole), $1.5 \times 10^{-3}$ M OH-urea and $10^{-6}$ M 5 - fluoro - 2' deoxyuridine (FdUrd). The cells were then harvested, washed and lysed and the DNA was extracted by a modification of a method described by Cleaver (1968).

Cells were harvested and washed 3 times with PBS as described in methods section 2.1.5. The pellet was then treated with 3.0 ml 0.1% (w/v) sodium dodecyl sulphate (SDS) in saline-citrate buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The cell lysates were subjected to 3 rapid freeze thaw cycles, incubated with RNase - A (50 $\mu$g/ml) for an hour; then with pronase (500 $\mu$g/ml) for a further 2 hours at 37°C and finally deproteinised by shaking gently 3 times with equal volumes of chloroform - amyl alcohol (24 : 1 v/v). After centrifuging at 1000 g at room temperature for 10 min, the aqueous layers were collected and made up to 3.5 ml with saline-citrate buffer. Solid caesium chloride was added to a final concentration of 55.2% (w/v) and the resulting solutions centrifuged at 37,000 rpm for 40 h in a Beckman spinco 50 Ti, angle rotor, at room temperature. After centrifugations, the tubes were decapped and six-drop fractions were collected, using a peristaltic pump, into tubes containing 0.3 ml distilled water.

The extinction of each fraction was determined at 260 nm using a Cecil CE 212 variable wavelength u.v. spectrophotometer. The fractions

were then mixed with 10% ice-cold TCA (1 : 1) to give a final TCA concentration of 5% and filtered through glass fibre filter (Whatman 2.5 cm diameter), washed 4 times with 1.0 ml cold 5% TCA, once with 2.0 ml methylated spirit and placed in a scintillation vial. The vials were dried for at least 2 h at 60°C. They were processed for counting and counted as described in methods section 2.1.6.

2.1.9 Analysis of the dT nucleotide pools in lymphocytes

Cultures of $3.0 \times 10^6$ lymphocytes suspended in 1 ml [illegible], were irradiated at 20 $J.m^{-2}$. 3.0 ml RPMI were added containing [$^3$H] - dT (final concentration 5 $\mu$Ci/ml, 21 Ci/mmole) and OH-urea ($1.5 \times 10^{-3}$ M final concentration). Unlabelled dT was also added as required to produce dT specific activity diluted by a factor 2, 4 and 8. All cultures were incubated at 37°C for 4 h. The cells were harvested and washed 4 times with 5.0 ml ice-cold [illegible] (see materials section 2.2.4) to remove all the labelled nucleoside. The efficiency of the washing procedure was checked by counting a 1.0 ml sample of the supernatant [illegible] after each cell wash in triton-toluene liquid scintillation fluid (see materials section 2.2.4).

To determine the specific activity of dT nucleotide (acid-soluble) pools, the cells were washed twice (2 x 1.0 ml) with ice-cold 5% TCA for 15 min and 5 min respectively. The two extracts for each culture were combined. 1.0 ml aliquots of the acid-soluble extracts were added to scintillation vials containing 10 ml of triton-toluene liquid scintillation fluid and counted.

The incorporation of [$^3$H] - dT into the acid-insoluble fraction was estimated by transferring the TCA-precipitate to a Whatman glass fibre filter (2.5 cm diameter), washing at least 4 times with ice-cold 5% TCA and then once with 2.0 ml methylated spirit. The filters were placed in scintillation vials, allowed to dry at 60°C for 2 h and counted as described in methods section 2.1.6.

## 2.2 Materials

### 2.2.1 Chemicals

Amfix was obtained from May & Baker, Dagenham, England.

Caesium Chloride (Analar grade) was obtained from Hopkin & Williams Ltd., Chadwell Heath, Essex, England.

DePex and Giemsa stain were obtained from George T. Gurr Ltd., London.

2,5 - diphenyloxazole (PPO) and toluene were obtained from Koch-Light Laboratories, Colnbrook, Bucks., England.

Ficoll - 400 was obtained from Pharmacia (G.B.) Ltd., Paramount House, London.

5 - Fluorodeoxyuridine and Pronase (B grade) were obtained from Calbiochem, Los Angeles, California, U.S.A.

Heparin was obtained from Evans Medical Ltd., Speke, Liverpool, England.

Hyamine hydroxide (1 M solution in methanol) was obtained from Nuclear Enterprises Ltd., Edinburgh.

Hydroxyurea was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

Ilford L4 Nuclear research emulsion (in gel form, size A) was obtained from Ilford Ltd., Ilford, Essex, England.

Metol was obtained from Kodak Ltd., London

Phytohaemagglutinin (PHA) (reagent grade, bottle contains 5 ml freeze dried PHA, reconstituted in 5.0 ml water), was obtained from Wellcome Reagent Ltd., Wellcome Research Laboratories, Bucks, England.

Potassium Ferrioxalate $K_3Fe(C_2O_4)_3\ 3H_2O$ was prepared by mixing 3 volumes of 1.5 M potassium oxalate and 1 volume of 1.5 M ferric chloride as described by Hatchard and Parker (1956). The potassium ferrioxalate was recrystalized three times from warm water and air dried in the dark.

Sodium Metrizoate 3 - acetamido - 2, 4, 6 - triiodo - 5 - (N - methylacetamido) - benzoic acid , molecular weight is 649.9, delivered at a concentration of 32.8% (w/v) and a density of 1.200 g/ml at 20°C was obtained from Nyegaard & Co., As, Oslo, Norway.

### 2.2.2 Cell culture reagents

Amino acids, vitamins and foetal calf serum were obtained from Bio-Cult Laboratories, Glasgow.

Penicillin and Streptomycin were obtained from Glaxo Laboratories Ltd., Greenford, Middlesex, England.

### 2.2.3 Radiochemicals

Methyl - [$^3$H] - thymidine and 6 - [$^3$H] - 5 - bromo - 2'deoxyuridine were obtained from the Radiochemical Centre, Amersham, Bucks., England.

### 2.2.4 Solutions & buffers

Actinometer buffer — was prepared by mixing 600 ml 1 M - sodium acetate, 360 ml 0.5 M - $H_2SO_4$ and 40 ml water.

Amfix — was prepared by diluting stock 1 : 5 with water (v/v).

Carnoy's fixative — was prepared by mixing glacial acetic acid and absolute ethanol in the ratio 1 : 3

| | |
|---|---|
| D19b developer | composition is shown in Table 2 |
| Eagle's medium | composition is shown in Table 4 |
| Giemsa stain | contained 0.75% Giemsa in glycerol-methanol (1 : 1 v/v) |
| Lymphocyte separation fluid | contained 29.4 ml sodium metrizoate made up to 100 ml with 8% (w/v) FiColl 400 |
| Phosphate Buffered Saline (PBS) | was composed of solutions A, B and C shown in Table 3 in the ratio 8 : 1 : 1 respectively. |
| Potassium ferrioxalate solution | was prepared by dissolving 2.947 g of $K_3Fe(C_2O_4)_3\ 3H_2O$ in 800 ml water, adding 100 ml 0.5 M $H_2SO_4$ and making the solution up to 1.0 l with water. |
| Saline-citrate buffer | contained 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0 |
| Toluene-based scintillation fluid | contained 0.5% 2,5 diphenyloxazole (PPO) in A.R. toluene |
| Triton-toluene scintillation fluid | consisted of PPO, 5 g/l and Bis MSB, 0.5 g/l in Triton X-100 : toluene (1 : 1) |

2.2.5 Cell culture medium

($EFC_{15}$) Eagle's medium supplemented with 15% foetal calf serum. (for the composition of Eagle's medium see Table 4 ).

Table 2

D19b developer

| | |
|---|---|
| $Na_2SO_3 \cdot 7H_2O$ | 144 g |
| $Na_2CO_3$ | 48 g |
| KBr | 4 g |
| Hydroquinone | 8.8 g |
| Metol | 2.2 g |
| Total volume | 1 litre |

Table 3

Phosphate compound saline (PBS)

Solution A in a total volume of 1 litre

| | |
|---|---|
| NaCl | 10 g |
| KCl | 0.25 g |
| $Na_2HPO_4$ | 1.44 g |
| $KH_2PO_4$ | 0.25 g |

Solution B in a total volume of 1 litre

| | |
|---|---|
| $CaCl_2 \cdot 2H_2O$ | 1.0 g |

Solution C in a total volume of 1 litre

| | |
|---|---|
| $MgCl_2 \cdot 6H_2O$ | 1.0 g |

Table 4

Eagle's medium

| | |
|---|---|
| NaCl | 6.8 g |
| KCl | 0.4 g |
| $CaCl_2 \cdot 6H_2O$ | 0.393 g |
| $MgSO_4 \cdot 7H_2O$ | 0.2 g |
| $NaH_2PO_4 \cdot 2H_2O$ | 0.14 g |
| $NaHCO_3$ | 2.2 g |
| Glucose | 4.5 g |
| L-arginine.HCl | 0.0421 g |
| L-cystine | 0.0240 g |
| L-glutamine | 0.2920 g |
| L-Histidine.HCl | 0.0192 g |
| L-isoleucine | 0.0525 g |
| L-leucine | 0.0525 g |
| L-leucine.HCl | 0.0731 g |
| L-methionine | 0.0149 g |
| L-phenylalanine | 0.0330 g |
| L-threonine | 0.0476 g |
| L-tryptophan | 0.0082 g |
| L-tyrosine | 0.0362 g |
| L-valine | 0.0469 g |
| D-calcium pantothenate | 0.002 g |
| Choline chloride | 0.002 g |
| Folic acid | 0.002 g |
| i-inositol | 0.004 g |
| Nicotinamide | 0.002 g |
| Pyridoxal.HCl | 0.002 g |
| Riboflavin | 0.0002 g |
| Thiamine.HCl | 0.002 g |
| Penicillin | 100,000 units |
| Streptomycin | 0.1 g |
| Phenol red | 0.015 g |
| Total volume | 1 litre |

# 3. RESULTS

## 3.1 Introduction

The principal methods used in the present project to measure DNA repair after u.v. irradiation of lymphocytes derived from AK patients and age matched normal individuals were:-

1) Autoradiography of cells after u.v. irradiation

The use of [$^3$H] - dT incorporation and autoradiography has resulted in major advances in the study of DNA synthesis and the related problems of cell proliferation (e.g. Taylor et al 1957; Baserga and Malamud 1969; Dormer 1973). This approach has also been used to study DNA repair.

More than 99% of peripheral lymphocytes are non-dividing cells (see methods section 2.1.6) and do not incorporate [$^3$H] - dT when incubated with the labelled nucleoside in culture (see Plate 4). However, lymphocytes which have been irradiated with u.v. light begin to incorporate [$^3$H] - dT as DNA repair commences. This incorporation can be detected and measured by counting grains over cells' nuclei after autoradiography (see Plate 5). The few cells in S-phase (which are only very rarely seen in autoradiography) can be readily distinguished by their much greater grain density and are not included in the repair assay.

Although this method is time consuming (i.e. autoradiographic exposure and grain counting), it is a well established and unambiguous procedure for estimating DNA repair. It has been used successfully in the estimation of DNA repair*fibroblasts (Cleaver 1968) and in human lymphocytes (Evans and Norman 1968; Frey-Wettstein et al 1969).

* in

Plate 4 Autoradiograph of normal unirradiated lymphocytes incubated with [$^3$H] - dT for 4 h at 37°C. There are essentially no grains over the cells nuclei. For experimental details see the legend to Fig. 9.

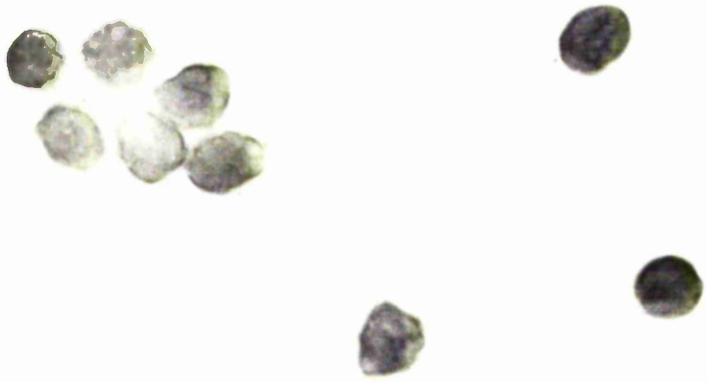
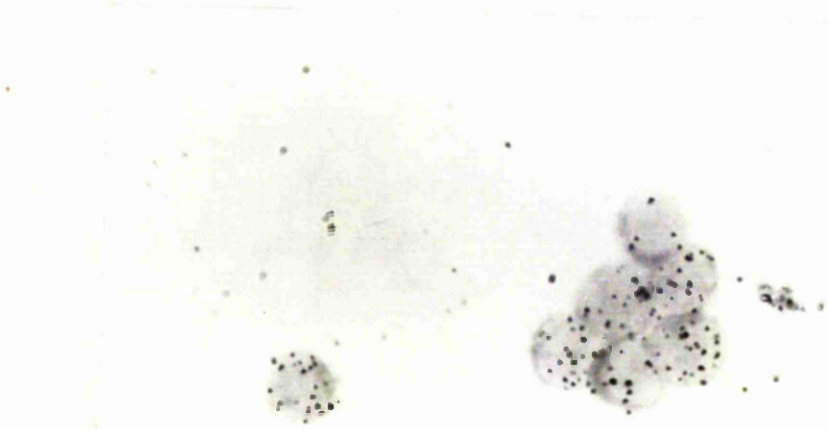


Plate 5 Autoradiograph of normal lymphocytes exposed to 5.0 $J.m^{-2}$ and incubated with $[^3H]$ - dT for 4 h at $37^{\circ}C$. All cells show a recognizable grain count. For experimental details see the legend to Fig. 9



2) Total incorporation of $[^3H]$- dT into cells after u.v. irradiation

$[^3H]$- dT is extensively incorporated into dividing cells in culture during the S-phase of the cell cycle. If such cells are irradiated with u.v. light before labelling, the relatively small incorporation of $[^3H]$- dT due to u.v. induced DNA repair is completely masked by that due to replication. In lymphocyte cultures however, only a few cells (<1%) are dividing (see methods section 2.1.6) and hence the rate of $[^3H]$- dT incorporation into replicating DNA is very low. Nevertheless, even this low level of DNA synthesis is greater than and overshadows u.v. induced DNA repair synthesis in human lymphocytes. However, if DNA replication in the few cells in S-phase is inhibited by adding OH-urea to the medium, then repair synthesis becomes the predominant cause of $[^3H]$- dT incorporation into DNA. The OH-urea inhibits S-phase DNA synthesis without affecting repair (Cleaver 1969a). Under these conditions the difference in total incorporation of $[^3H]$- dT between u.v. irradiated and unirradiated lymphocytes can be used as a direct measure of DNA repair. This approach had only been mentioned in one brief report (Robbins et al 1970) before the start of this work but has subsequently been described (Lambert et al 1976a).

3) Recovery of DNA replication after u.v. irradiation

When lymphocytes are treated with PHA most of them (~80%) are stimulated into a division cycle. DNA replication commences approximately 30 h after PHA treatment and reaches a peak 18 h later (see Fig. 23). U.v. irradiation of growing cells damages the DNA and consequently inhibits replication

(Cleaver 1967; Painter et al 1970). However, as the DNA damage is repaired, DNA replication resumes (Rasmussen et al 1970). The rate of recovery of DNA replication after u.v. irradiation has been used in this work as a novel approach for the study of DNA repair. The other two methods only measure the extent of repair synthesis and do not indicate the quality of the products. This method however, depends on the quality of the repair process in that it must be sufficiently accurate to allow the recovery of DNA replication.

The first part of the results section deals with establishing the autoradiographic and total incorporation methods of estimating DNA repair in lymphocytes. This is followed by an examination of the effect of patient age on DNA repair which is important in view of the fact that AK patients are usually elderly (see Introduction Section 1.6). DNA repair activity is then examined in lymphocytes derived from AK patients and age-matched normal individuals.

Results are then presented which confirm that the u.v. induced $[^3H]$- dT incorporation into lymphocytes in the presence of OH-urea is due to DNA repair synthesis and not to some form of u.v.-stimulated DNA replication and to show that differences in u.v.-induced $[^3H]$ - dT incorporation in the cell types used are not due to differences in the specific activity of the cellular $[^3H]$- dTTP caused by different dT-nucleotides pool sizes.

The next section presents a study of DNA repair in lymphocytes from AK and normal patients over longer time courses. This is followed by the results of the study of DNA repair in cells from

AK patients and age-matched normal subjects, based on the rate of recovery of DNA replication after u.v. irradiation.

The last section is a preliminary investigation of the state of u.v. induced DNA repair in some conditions other than AK.

## 3.2 The use of [$^3$H] - dT and autoradiography to study DNA synthesis

The specificity of incorporation of dT into DNA and the high autoradiographic resolution of tritium provide a convenient combination for the investigation of DNA synthesis and associated problems of cell proliferation.

Autoradiographs (see Plate 5) of u.v. irradiated lymphocytes, labelled with [$^3$H] - dT show specific labelling of the cell nucleus with no grains above the background over the cytoplasm. Incorporation of [$^3$H] - dT into RNA or fixation of [$^3$H] - nucleoside mono-, di-, or triphosphate or other labelled substances have been shown to be insignificant by Amano et al (1959), who reported that all detectable incorporation (measured by grain counting) was removed by DNase treatment of mammalian cells, while RNase treatment had no effect.

## 3.3 Time course of DNA repair in human lymphocytes

The rate of [$^3$H] - dT incorporation into the acid insoluble fraction (TCA) of u.v. irradiated lymphocytes of both actinic and normal individuals was measured by the total incorporation method in the presence of CR-urea (see Methods Section 2.1.6).

The initial u.v. dose chosen to stimulate [$^3$H] - dT incorporation into DNA was 20 $J.m^{-2}$. This dose has been reported by various authors

(Evans and Norman 1968; Cleaver 1969a; Meneghini 1974) to give rise to maximum or near maximum DNA repair.

Fig. 8 shows that repair appears to be complete in normal cells 4 h after irradiation. This period is comparable to that (4-5 h) reported by Evans and Norman (1968) for human lymphocytes and is the same as that reported by Meneghini (1974) for opossum lymphocytes.

Although the rate of repair in AK lymphocytes is lower than that for lymphocytes from age-matched normal individuals at all the times examined (Fig. 8), it does not appear to have reached saturation at the end of the fourth hour. This observation poses the question, as to whether the difference in repair activity between the lymphocytes of AK patients and those of age-matched normal individuals is due to a slower rate of repair or to a lower total capacity for repair synthesis (e.g. shorter inserted pieces) of the former. This problem is examined in more detail later (see Results Section 3.10).

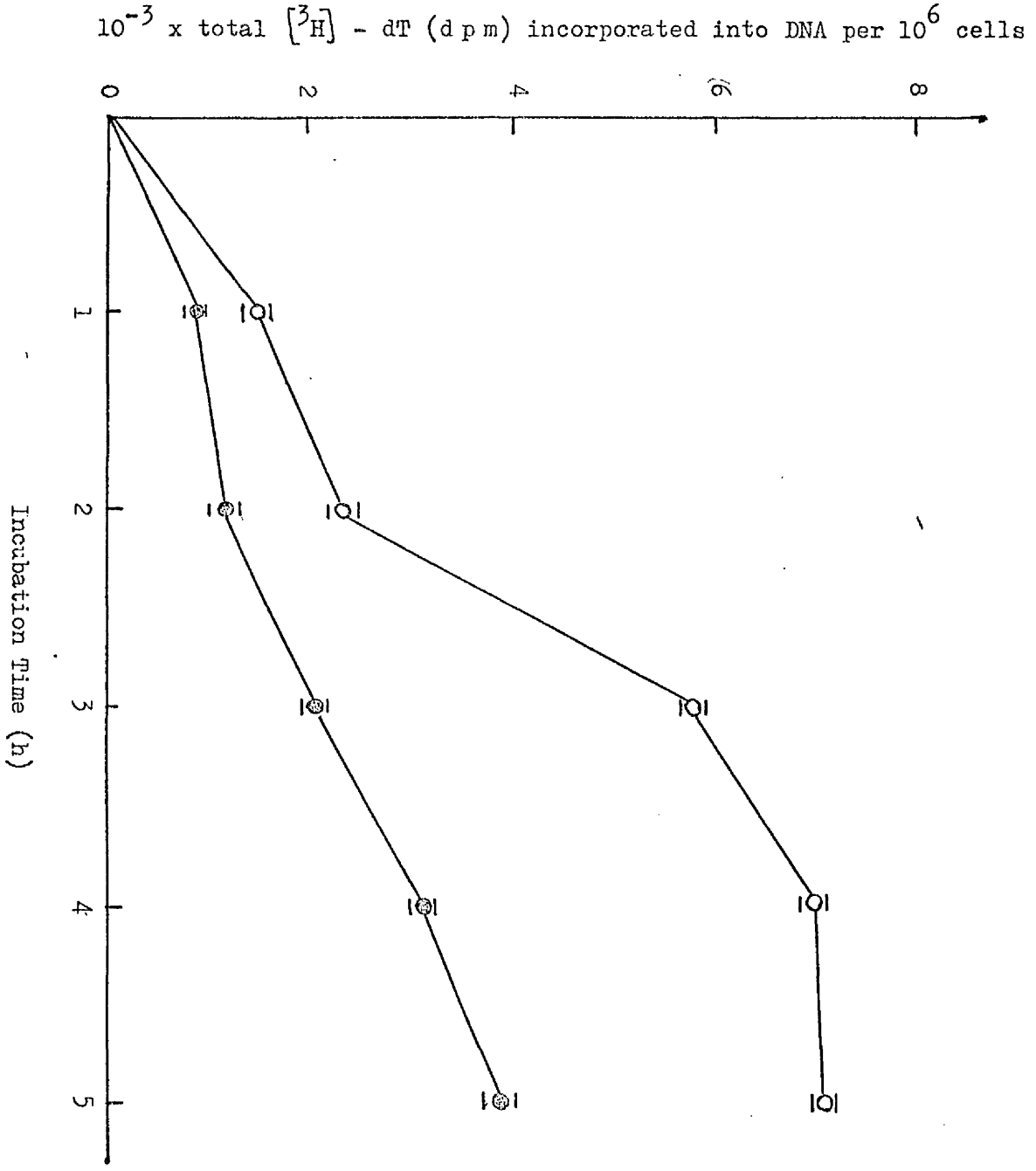
While other authors have arbitrarily selected 1 or 2 h post-irradiation period for the study of DNA repair (Cleaver 1968; Lambert et al 1976a), a 4 h period was chosen for the subsequent assays for the following reasons:-

1) This is the maximum time period over which the rate of repair (incorporation of $[^3H]$ - dT per unit time) appears to be approximately constant for both normal and AK cells.
2) Shorter incubation times would result in lower $[^3H]$ - dT incorporation into DNA and therefore reduce the accuracy of the estimations.

Fig. 8 Time course of $[^3H]$ - dT incorporation into DNA of u.v. irradiated lymphocytes derived from AK patients and age-matched normal individuals, measured by the total incorporation method. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3), were incubated immediately after irradiation (20 $J.m^{-2}$) with $[^3H]$ - dT (5 $\mu$Ci/ml, 18.5 Ci/mmole) and OH-urea ($1.5 \times 10^{-3}$ M) for different times from 0 to 5 h at 37°C. After incubation, the cultures were washed, fixed and assayed for $[^3H]$ - dT incorporation as described in methods section 2.1.6. Each point represents the mean of duplicate determinations in different cultures and the bars represent the ranges. The counting efficiency was determined by the channels ratio method.

○ Normal patients lymphocytes

● AK patients lymphocytes


$10^{-3}$ x total [$^3$H] - dT (d p m) incorporated into DNA per $10^6$ cells
0
2
4
6
8
Incubation Time (h)
1
2
3
4
5

3.4 U.V. dose response curves for DNA repair in human lymphocytes

The effect of u.v. dose on the rate of DNA repair was examined in normal and AK lymphocytes using the autoradiographic method. Fig. 9 shows that the response to increasing u.v. dose of DNA repair in both types of lymphocyte is non-linear except perhaps at very low doses. This result is similar to that presented by Cleaver & Bootsma (1975) for normal human fibroblasts using the same method which shows the response approaching a plateau in a similar way at u.v. doses of about 20 - 25 $J.m^{-2}$. Any slight difference in u.v. dose required for repair to reach saturation in fibroblasts compared with lymphocytes could be due to the relatively smaller cytoplasmic RNA content of non-dividing lymphocytes as opposed to the dividing fibroblasts (Cooper and Rubin 1965). The higher RNA content of the cell cytoplasm could provide a more effective shield for the nucleus by absorbing more u.v. irradiation.

3.5 The similarity of u.v. induced DNA repair in human lymphocytes from normal individuals of different ages

Actinic Keratosis is a pre-neoplastic condition which occurs mostly in people over 50 years of age (Grinspan and Abulafia 1955; Rook 1956; Pinkus 1971), so it was important to assess the effect age might have on the level of DNA repair in human lymphocytes. Repair was measured using the total incorporation method in the presence of OH-urea in lymphocytes of normal individuals between 17 - 77 years of age. The results obtained (see Fig. 10) show that there is no detectable change in DNA repair over this age range.

Fig. 9 Dose response curves of u.v. induced DNA repair in lymphocytes derived from AK patients and age-matched normal individuals measured by autoradiography. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3) were treated immediately after u.v. irradiation at different doses ranging from 0 to 20 $J.m^{-2}$, with [$^3$H] - dT (5 $\mu$Ci/ml, 18.5 Ci/mmole) for 4 h at 37°C. The cultures were washed, fixed and processed for autoradiography as described in methods section 2.1.5. The autoradiographs were exposed for 8 days. Silver grains were counted over the nuclei of 150 - 200 cells (but not over the very rare S-phase cells). Each point represents the mean grain count/cell of duplicate determinations in different cultures minus the mean value of that in the corresponding unirradiated cultures and the bars represent the ranges. Less than 1% of the cells were in S-phase.

○ Normal lymphocytes

● AK lymphocytes

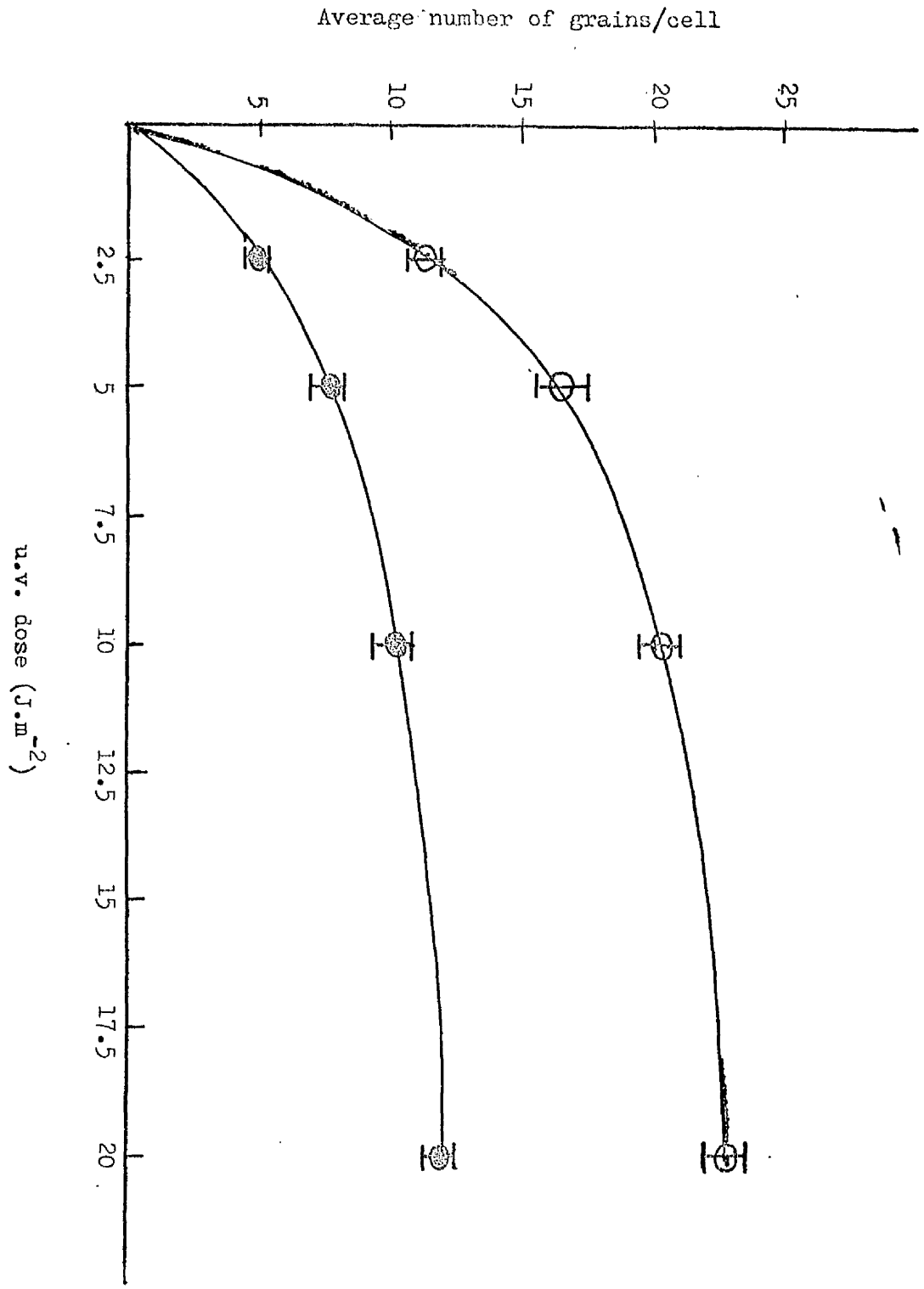
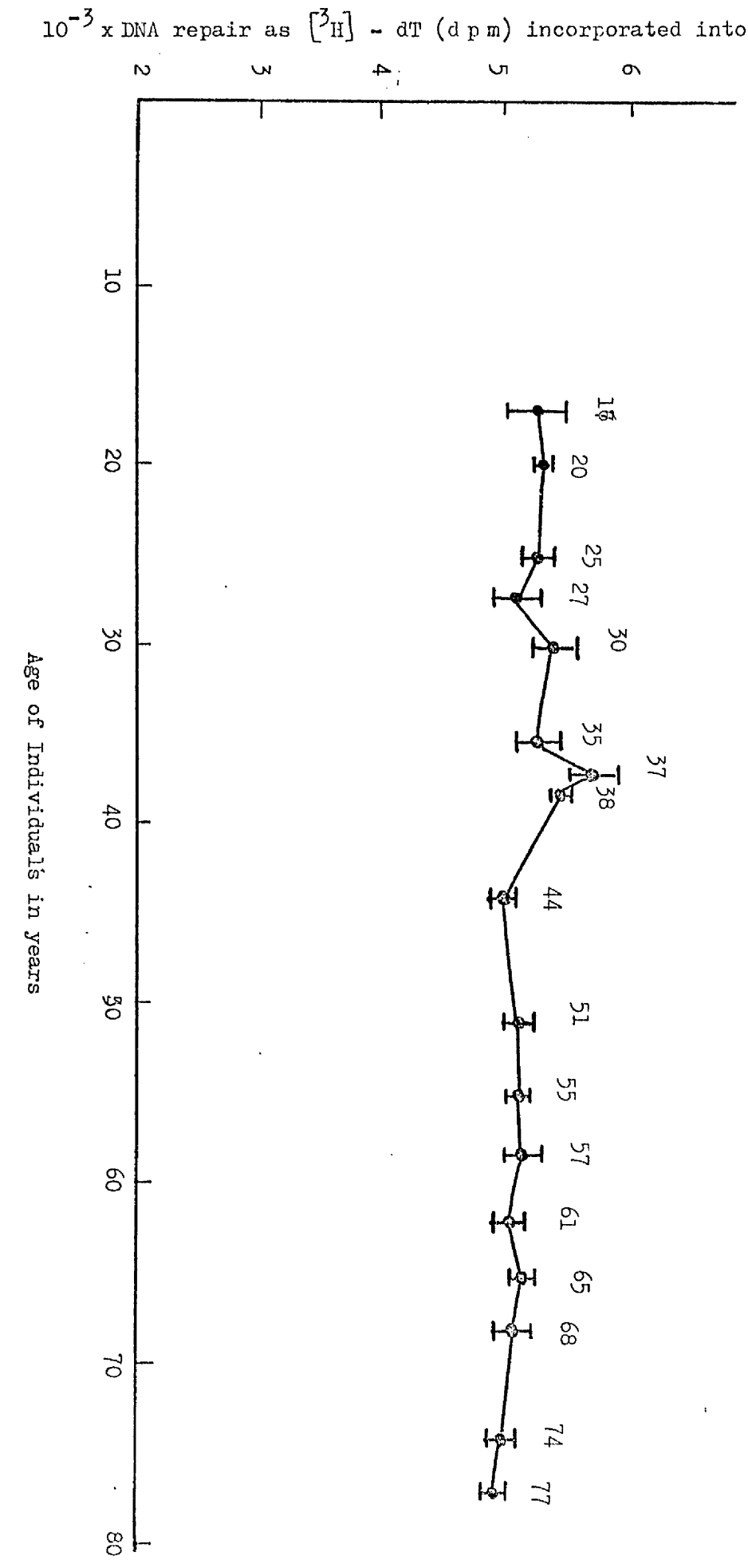

Average number of grains/cell
5
10
15
20
25
2.5
5
7.5
10
12.5
15
17.5
20
u.v. dose (J.m$^{-2}$)

Fig. 10 Variation of DNA repair with age, in lymphocytes derived from normal individuals, measured by the total incorporation method. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3) were incubated immediately after irradiation (20 $J.m^{-2}$) or mock irradiation with [$^3$H] - dT (5 $\mu$Ci/ml, 18.5 Ci/mmole) and OH-urea ($1.5 \times 10^{-3}$ M) at 37°C for 4 h. After incubation, cultures were washed, fixed and assayed for [$^3$H] - dT incorporation as described in methods section 2.1.6. Each value was obtained by subtracting from the d.p.m for each irradiated culture, the mean of d.p.m for duplicate unirradiated controls of the same donor. Points represent the means of duplicate determinations in different cultures and bars represent the ranges. The counting efficiency was determined by the channels ratio method.


$10^{-3}$ x DNA repair as $[^3H]$ - dT (d p m) incorporated into $10^6$ cells
2
3
4
5
6
10
20
30
40
50
60
70
80
16
20
25
27
30
35
37
38
44
51
55
57
61
65
68
74
77
Age of Individuals in years

3.6 Autoradiographic comparison of u.v. induced DNA repair in lymphocytes derived from AK patients and age-matched normal individuals.

The lymphocyte system is ideal for the study of the u.v.-stimulated DNA repair by autoradiography. There are virtually no replicating cells, so partially u.v. inhibited S-phase cells (Bootsma and Humphrey 1968) can not be confused with non S-phase cells engaged in DNA repair. This can present a problem in the interpretation of autoradiographs of u.v. irradiated fibroblast cultures (Papasarantopoulou 1976).

Unstimulated lymphocytes do not show any incorporation of label when incubated with [$^3$H] - dT and examined autoradiographically (Plate 4), while all the cells incorporate the labelled nucleoside after u.v. irradiation (Plate 5).

Table 5 shows the u.v. stimulated DNA repair synthesis measured autoradiographically at 2 different u.v. doses in lymphocytes derived from 4 AK patients and the same number of age-matched normal individuals. There are essentially no grains (i.e. $< 0.2$ grain/cell) over the nuclei of unirradiated lymphocytes. The percentage of cells in S-phase never exceeded 1% and under these conditions they were readily distinguished from other cells because they appeared black and contained an uncountable number of grains. This is in accord with results presented by other workers (Bond et al 1958; Evans and Norman 1968; Frey-Wettstein et al 1969).

The grain counts over the u.v. stimulated AK and normal lymphocytes fall into Poisonion distributions (see Figs 11 & 12).

Table 5 U.v. induced DNA repair in lymphocytes derived from 4 AK patients and the same number of age-matched normal individuals, measured autoradiographically. Setting up the cultures, treatment of the cells and analysis of the results were the same as described in the legend to Fig. 9. Unirradiated cultures were essentially free of grains and the % S-phase cells was $< 1\%$ in all determinations. For each determination, the standard deviation and the standard error of the mean were worked out.

| Blood type | Mean grain count per cell irradiated at | | |
|---|---|---|---|
| | 0 $J.m^{-2}$ | 5.0 $J.m^{-2}$ | 20 $J.m^{-2}$ |
| Actinic AK6 | 0.180 | 8.80 ± 0.36 | 12.6 ± 0.64 |
| Normal NL6 | 0.08 | 17.60 ± 0.59 | 23.60 ± 0.93 |
| Actinic AK7 | 0.05 | 7.90 ± 0.40 | 11.40 ± 0.54 |
| Normal NL7 | 0.06 | 16.30 ± 0.69 | 25.12 ± 0.94 |
| Actinic AK8 | 0.08 | 8.20 ± 0.42 | 13.20 ± 0.58 |
| Normal NL8 | 0.12 | 18.30 ± 0.67 | 30.00 ± 1.00 |
| Actinic AK9 | 0.10 | - | 11.90 ± 0.57 |
| Normal NL9 | 0.08 | - | 21.20 ± 0.70 |

Fig. 11 Histogram showing the numbers of grains over the nuclei of lymphocytes derived from an AK patient. The experimental details are described in the legend of Fig. 9.

Fig. 12 Histogram showing the numbers of grains over the nuclei of lymphocytes derived from a normal individual. For experimental details see Fig. 9.

Fig. 13 Histogram showing the numbers of grains over the nuclei of mixed 1 : 1 population of lymphocytes derived from an AK patient and age-matched normal individual. For experimental details see Fig. 9.

Fig. 11

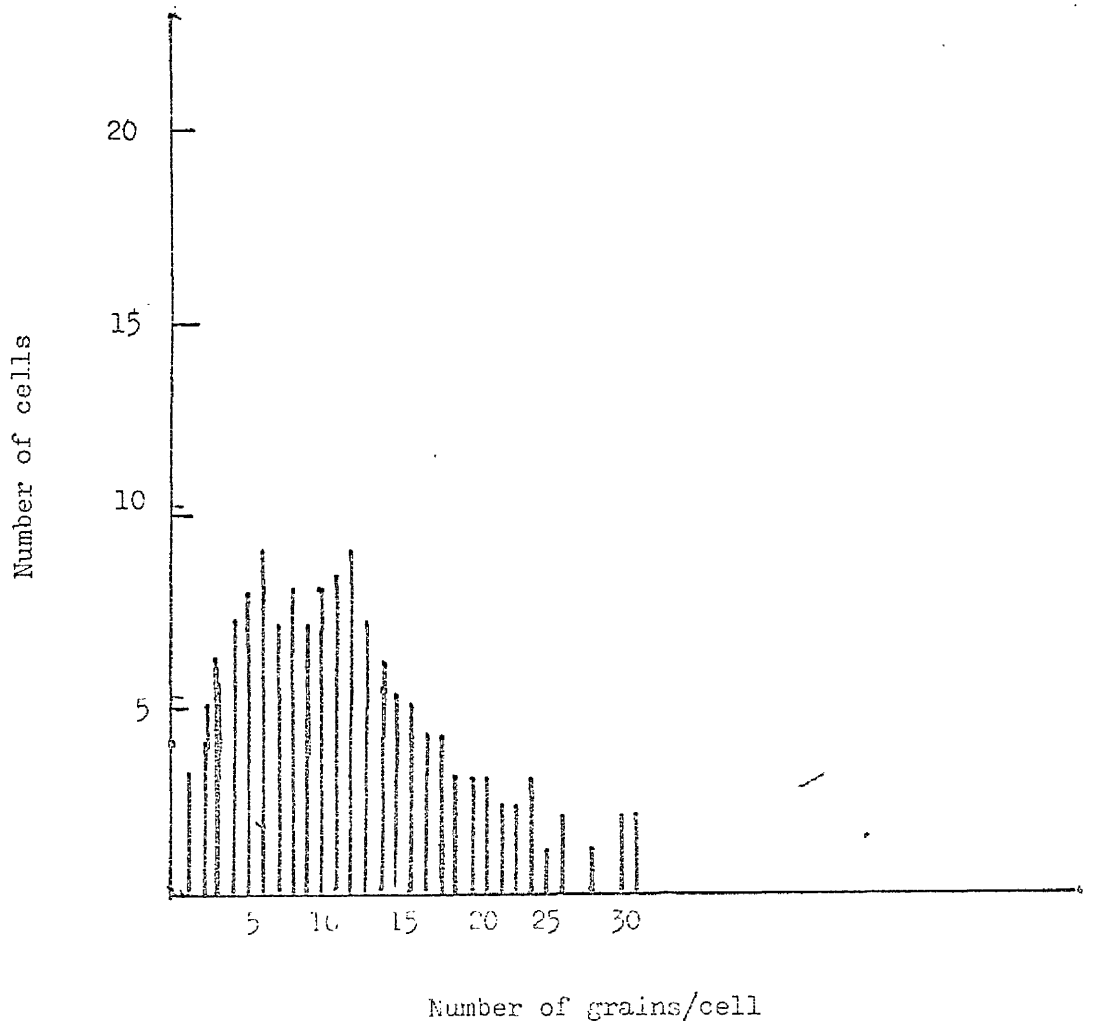


Fig. 12

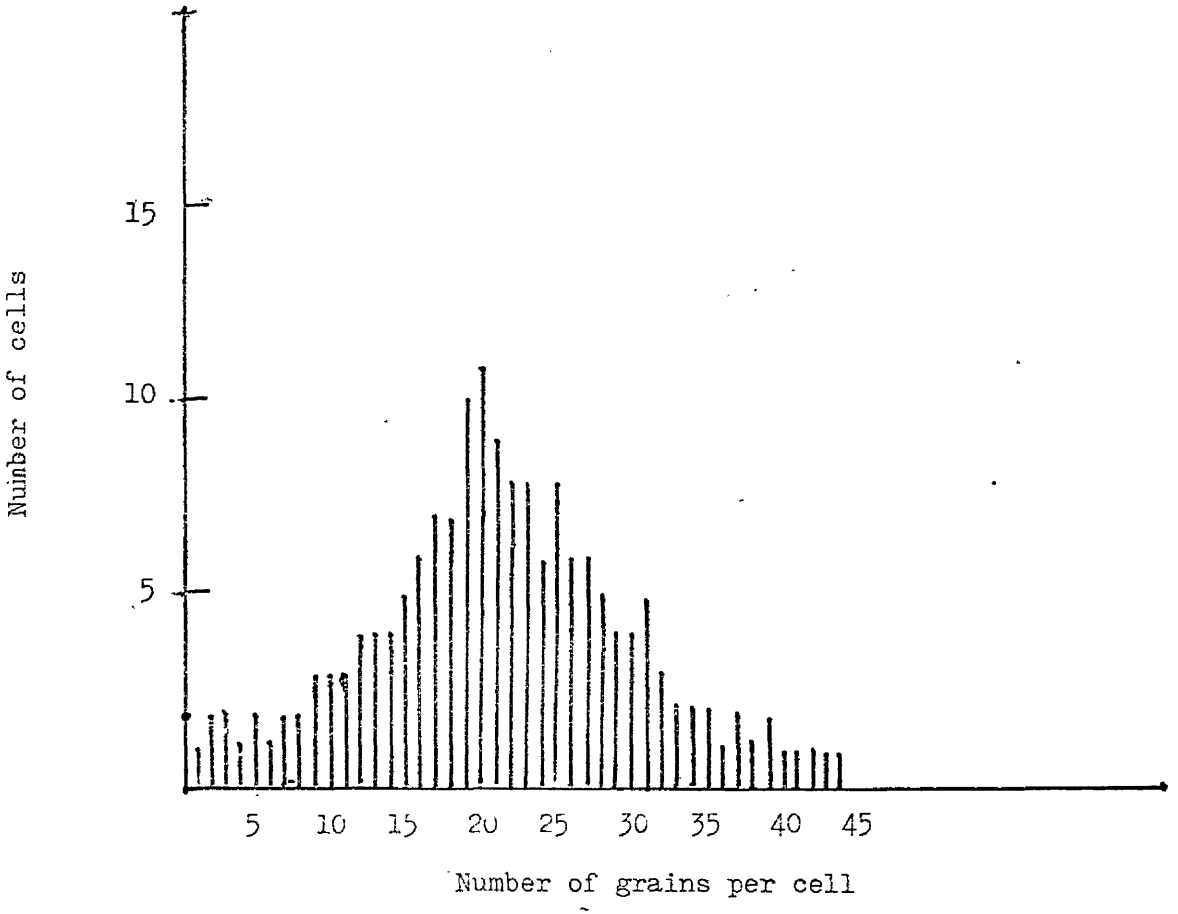
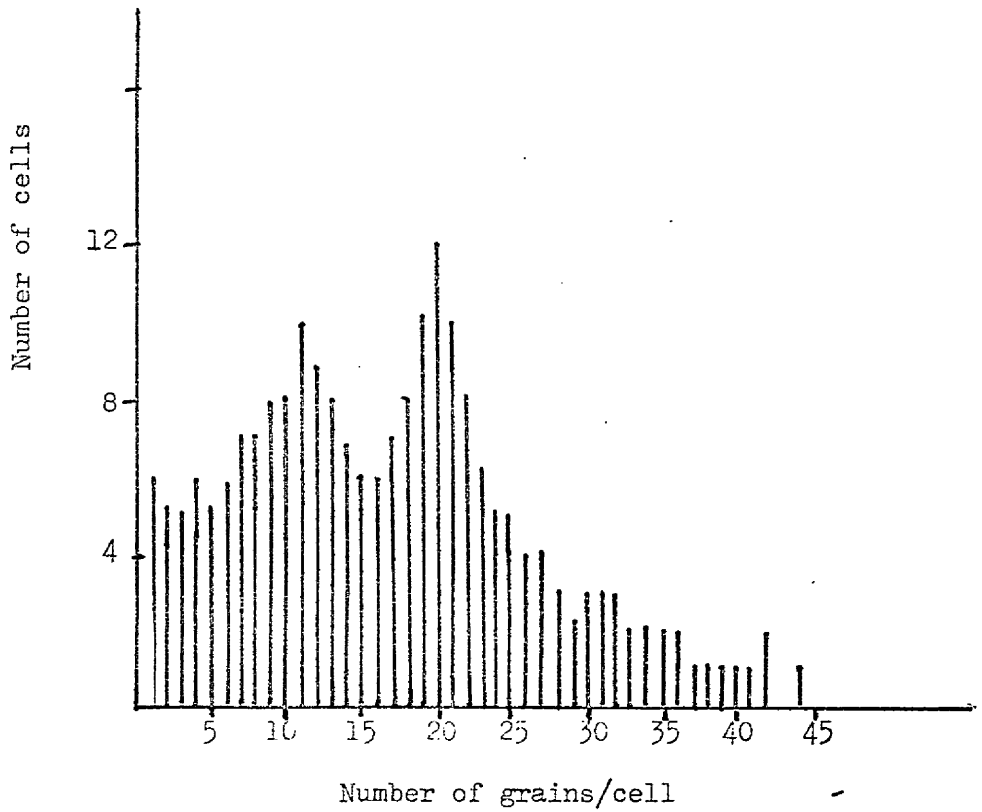

Number of cells
15
10
5
5
10
15
20
25
30
35
40
45
Number of grains per cell

Fig. 13


Number of cells
12
8
4
5
10
15
20
25
30
35
40
45
Number of grains/cell

The results presented in Table 5 show that the average u.v. stimulated DNA repair synthesis in AK lymphocytes is 47.4% and 48.7% that of the normal lymphocytes at doses of 5.0 and 20 $J.m^{-2}$ respectively. These values of u.v. induced DNA repair for AK and normal lymphocytes are very close to those obtained earlier when examining the u.v. dose responses for these cell types (Fig. 9).

The statistical significance of this difference was assessed with the Null hypothesis and student's t-test procedures. The value of t was calculated from the relation:-

$$t = \frac{\text{difference of the arithmetic means}}{\text{standard error of the difference of the means}}$$

The standard error of the difference of the means is given by $\sqrt{\frac{S_1^2}{n_1-1} + \frac{S_2^2}{n_2-1}}$, where $S_1$ and $S_2$ are the standard deviations from the mean for AK and normal lymphocyte populations, and $n_1$ and $n_2$ are the number of cells counted from each population. t gives a measure of the probability that the two populations are the same; the larger the values of t the smaller is the probability that the two populations are identical. Knowing the value of t and the number of independent observations (degrees of freedom), the probability P that the assumption made in the Null hypothesis is correct, can be obtained from statistical tables. Table 6 is a reconstruction of Table 5 with the values of t and P for each set of determinations inserted. Since the probability in each case is 0.001, it can be said with 99.9% confidence that the observed DNA repair values for AK and normal lymphocytes are different.

Table 6 U.v. induced DNA repair in lymphocytes derived from 4 AK patients and the same number of age-matched normal individuals, measured autoradiographically. Setting the cultures, treatment of the cells and analysis of the results were the same as described in the legend to Fig. 9. The values shown are the gross mean grain per cell in irradiated cultures recorded in Table 5 minus the values of their corresponding unirradiated controls. For each set of determinations comprising 1 AK and 1 normal irradiated at the same u.v. dose, the value of t (see text) was calculated and the probability of the two populations being the same was inserted.

| Blood type | Mean grain count per cell irradiated at 5.0 J.m$^{-2}$ | t calculated | t obtained from tables | Probability P | Mean grain count per cell irradiated at 20 J.m$^{-2}$ | t calculated | t obtained from tables | Probability P |
|---|---|---|---|---|---|---|---|---|
| AK6 | 8.62 ± 0.36 | 11.56 | 3.29 | < 0.001 | 12.42 ± 0.64 | 10.27 | 3.29 | < 0.001 |
| NL6 | 17.52 ± 0.59 | | | | 23.52 ± 0.93 | | | |
| AK7 | 7.85 ± 0.40 | 10.25 | 3.29 | < 0.001 | 11.35 ± 0.54 | 12.23 | 3.29 | < 0.001 |
| NL7 | 16.24 ± 0.69 | | | | 25.06 ± 0.94 | | | |
| AK8 | 8.12 ± 0.42 | 11.60 | 3.29 | < 0.001 | 13.12 ± 0.58 | 12.40 | 3.29 | < 0.001 |
| NL8 | 18.18 ± 0.67 | | | | 29.88 ± 1.00 | | | |
| AK9 | - | | | | 11.80 ± 0.57 | 10.48 | 3.29 | < 0.001 |
| NL9 | - | | | | 21.12 ± 0.70 | | | |

57

In order to reduce any subjective influence on the grain counting over cells from different populations, and to ensure that both populations of cells were treated under identical conditions, autoradiographs of mixed AK and normal lymphocytes irradiated and labelled as before were prepared and analysed. Fig. 13 shows a histogram of the grain counts of a 1 : 1 mixture of AK and normal lymphocytes. Two partially resolved peaks are evident, which are in similar positions to the individual peaks obtained from AK and normal lymphocytes analysed in separate cultures (see Figs 11 & 12).

The means of the grain counts over all the cells in mixed 1 : 1 populations of AK and normal lymphocytes in two experiments are very close (17.3 compared to 17.6; 18.8 compared to 18.7 respectively) to the average values of the means for the two cell types cultured separately (see Table 7).

The conclusions which can be drawn from the autoradiographic experiments so far described are:-

1) Both AK and normal lymphocytes show clearly detectable and easily measurable u.v. induced $[^3H]$ - dT incorporation (i.e. DNA repair).
2) The rate of $[^3H]$ - dT incorporation after u.v. irradiation into lymphocytes is 48 $\pm$ 4.8% of that into normal lymphocytes. This value represents the average of all experiments using a 4 h incubation period with $[^3H]$ - dT at all u.v. doses used (2.5 - 20 $J.m^{-2}$).

The simplest interpretation of this result is that the different levels of $[^3H]$ - dT incorporation into u.v. irradiated

Table 7 U.v. induced DNA repair in mixed 1 : 1 populations of lymphocytes derived from AK patients and age-matched normal individuals, measured autoradiographically. Cultures of mixed 1 : 1 populations of AK and normal cells were set up as described in methods section 2.1.3. The treatment of cells and the analysis of results were the same as described in the legend to Fig. 9. Unirradiated cultures were free of grains and were therefore not included in the table. For each determination, the standard deviation and the standard error of the mean were worked out.

| Experiment | Mean grain count per cell irradiated at 20 $J.m^{-2}$ for | | |
|---|---|---|---|
| | Normal | Actinic | mixed 1 : 1 |
| 1 | 24.56 ± 0.89 | 10.70 ± 0.52 | 17.3 ± 0.70 |
| 2 | 26.63 ± 0.95 | 12.84 ± 0.62 | 18.8 ± 0.74 |

AK lymphocytes and similarly treated normal lymphocytes is due to different rates of DNA repair synthesis.

However, it is possible that the difference in incorporation rates is due to a difference in enzymic activity (e.g. thymidine kinase; E.C. 2.7.1.21) and a difference in the pool sizes of the thymidine nucleotides and not to a real difference in the rates of DNA repair. This alternative explanation represents a general problem which must always be considered when interpreting data on the incorporation of precursors to compare macromolecular synthesis in different cell types but one which has been overlooked by previous workers studying DNA repair. This problem is examined in more detail in Results Section 3.9.

## 3.7 Measurement of DNA repair by the total incorporation method

Thymidine is readily incorporated into cells which are synthesising DNA. It was shown to be a specific precursor of DNA, in rats (Reichard and Estborn 1951) and in chick embryos (Friedkin et al 1956). The small amount of [$^3$H] - dT incorporated into the cytoplasm of chick embryo fibroblasts, grown in vitro was shown to be due to mitochondrial DNA (Meyer and Ris 1967; Meyer 1967). In this work, autoradiographs of u.v. irradiated lymphocytes grown in the presence of [$^3$H] - dT showed the incorporation of label to be nuclear (see Plate 5). Incorporation of [$^3$H] - dT into RNA was shown not to occur (Fleq and Pavan 1947; Amano et al 1959). The incorporation of [$^3$H] - dT into cells grown in vitro in the presence of the label should therefore provide a direct measure of DNA synthesis.

For the measurement of the total incorporation of $[^3H]$ - dT into lymphocytes grown in the presence of the labelled nucleoside, a modification of the method described by Robbins et al (1970) was used. Cells which have been incubated with $[^3H]$ - dT in culture, were washed, fixed and treated with ice-cold 5% TCA (w/v) as described in methods section 2.1.6. The treatment of cells with TCA causes the release of the nucleotides pools into solution, while at the same time precipitates the DNA as part of the acid-insoluble fraction, which can be collected on to a Whatman glass fibre filter and assayed for $[^3H]$ - dT incorporation as described in methods section 2.1.6.

3.7.1 Effect of u.v. irradiation on $[^3H]$ - dT incorporation into lymphocytes

Evans and Norman (1968 ) showed that $[^3H]$ - dT is incorporated into human peripheral blood lymphocytes, after u.v. irradiation, by autoradiography. This experimental approach was modified as described below to provide a simple and rapid assay for the study of DNA repair. The assay has been used to study DNA repair in lymphocytes derived from AK patients and age-matched normal individuals and in few cases from patients with melanoma or basal cell carcinoma (see Results Section 3.12 ).

Due to the presence of a very few dividing cells (<1%) in peripheral blood lymphocytes (see Methods Section 2.1.6), the incubation of freshly prepared lymphocytes with $[^3H]$ - dT results in a low rate of incorporation of the labelled nucleoside. A small increase in DNA synthesis resulting from DNA repair occurring after u.v. irradiation should therefore be detectable above this

low background of DNA replication. The increase in [$^3$H] - dT incorporation into u.v. irradiated cells should be a measure of DNA repair.

This approach can not be applied to dividing cells such as skin fibroblasts, with which most of the early work on DNA repair was carried out (Cleaver 1968). This is due to the much greater level of DNA replication (owed to the presence of greater proportion of S-phase cells) which completely masks DNA repair (Papasarantopoulu 1976).

The first attempt (Table 8) to measure u.v. induced [$^3$H] - dT incorporation into DNA of lymphocytes (by estimating the total incorporation of label instead of using autoradiographic analysis), showed only small and inconsistent increases in irradiated cells compared to unirradiated controls and there was no reproducible difference between the responses of AK and normal cells.

The small size of the increases in [$^3$H] - dT incorporation after u.v. irradiation appeared to be caused by the masking effects of the small but significant amount of S-phase DNA synthesis. To overcome this problem, use was made of the report by Cleaver (1969a) that the drug (OH-urea) inhibits S-phase DNA replication but not DNA repair. The reasons for this differential inhibition are not clearly understood and are discussed later (see Discussion).

Table 8 U.v. induced [$^3$H] - dT incorporation into lymphocytes derived from AK patients and age-matched normal individuals, measured by the total incorporation method in the absence of OH-urea. Cultures of 3 x $10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3), were incubated immediately after irradiation (0 - 20 J.m$^{-2}$) with $^3$H - dT (5 $\mu$Ci/ml, 18.5 Ci/mmole) at 37°C for 4 h. After incubation the cultures were washed, fixed and assayed for [$^3$H] - dT incorporation as described in methods section 2.1.6. Each value represents the mean c p m of duplicate determinations in different cultures. The counting efficiency was determined by the channels ratio method.

| Blood type | Total $[^3H]$ - dT incorporation (c p m) per $10^6$ cells irradiated at | | | |
|---|---|---|---|---|
| | 0 $J.m^{-2}$ | 2.5 $J.m^{-2}$ | 10.0 $J.m^{-2}$ | 20 $J.m^{-2}$ |
| AK 10 | 1810 | 1821 | 2015 | 1928 |
| NL 10 | 1876 | 1809 | 2012 | 2083 |
| AK 11 | 1724 | 1685 | 1829 | 1851 |
| NL 11 | 1904 | 1920 | 2053 | 1988 |

3.7.2 Effect of u.v. irradiation on $[^3H]$ - dT incorporation into lymphocytes in the presence of OH-urea

OH-urea was added to lymphocyte cultures to inhibit DNA replication in the few S-phase cells at a concentration ($1.5 \times 10^{-3}$M) similar to that used by Frey-Wettstein et al (1969) and Robbins and Kraemer (1972a). Under these conditions it was possible to measure differences in $[^3H]$ - dT incorporation between u.v. irradiated and unirradiated cells of the same type and to measure differences in response between AK and normal u.v. irradiated lymphocytes (see Table 9).

The u.v. dose response for AK and normal lymphocytes was also determined by measuring the total incorporation of $[^3H]$ - dT into OH-urea treated lymphocytes, irradiated at different u.v. doses and incubated with the label for 4 h at 37°C after incubation. The u.v. induced DNA repair was calculated by subtracting the incorporation of $[^3H]$ - dT into unirradiated cells from the incorporation into irradiated cells. The repair at each u.v. dose is higher in normal lymphocytes than in AK lymphocytes (Fig. 14)

Fig. 14 also shows that the u.v. dose response curves obtained by the total incorporation method plateau between 10 and 20 $J.m^{-2}$ for both AK and normal lymphocytes. However, the DNA repair calculated as described above is probably less than the true value especially at high u.v. doses due to the inhibition of DNA replication by u.v. light (Painter et al 1970). DNA replication occurs in only a small percentage (<1%) of lymphocytes (see Methods Section 2.1.6) but even in the presence of OH-urea, the incorporation

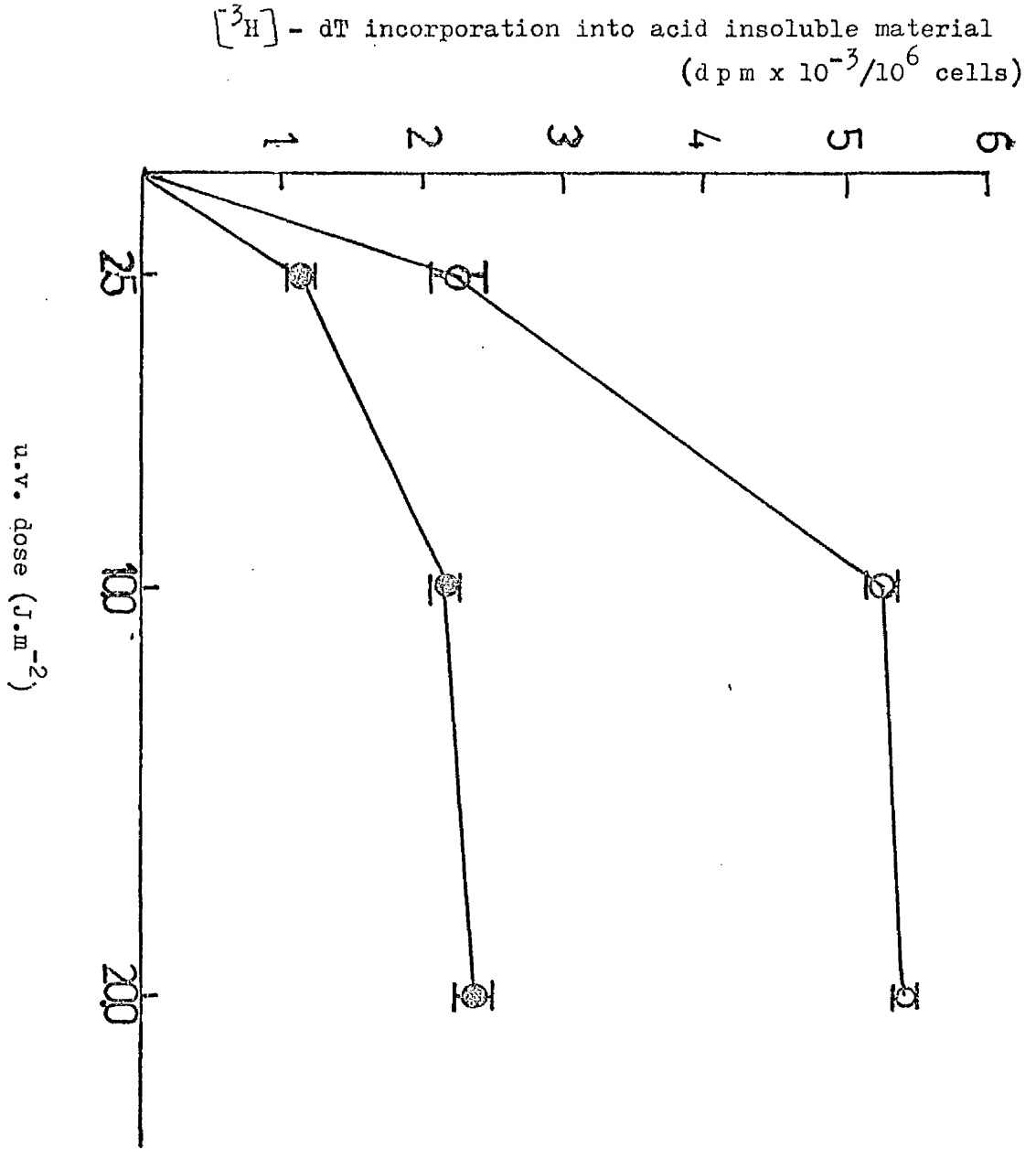
Table 9 U.v. induced DNA repair in lymphocytes derived from AK patients and age-matched normal individuals, measured by the total incorporation method in the presence of OH-urea. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3), were incubated immediately after irradiation (0 - 20 $J.m^{-2}$) with $[^3H]$ - dT (5 $\mu$Ci/ml, 18.5 Ci/mmole) and OH-urea ($1.5 \times 10^{-3}$M) at 37°C for 4 h. After incubation, the cultures were washed, fixed and assayed for $[^3H]$ - dT incorporation as described in methods section 2.1.6. Each value represents the mean d p m of duplicate determinations in different cultures and the brackets represent the minimum values for DNA repair (see text). The counting efficiency was determined by the channels ratio method.

| Blood type | Total $[^3H]$ - dT incorporation (d p m) per $10^6$ cells irradiated at | | | |
|---|---|---|---|---|
| | 0 $J.m^{-2}$ | 2.5 $J.m^{-2}$ | 10.0 $J.m^{-2}$ | 20 $J.m^{-2}$ |
| AK 12 | 1343 (0) | 2413 (1070) | 3630 (2287) | 3753 (2410) |
| NL 12 | 1320 (0) | 3366 (2046) | 6483 (5163) | 6872 (5522) |
| AK 13 | 1520 (0) | 2706 (1186) | 3833 (2313) | 3920 (2400 |
| NL 13 | 1393 (0) | 4143 (2750) | 6882 (5489 | 6306 (4913) |
| AK 14 | 1360 (0) | 2530 (1170) | 3940 (2580) | 4001 (2641) |
| NL 14 | 1330 (0) | 3733 (2403) | 7126 (5796) | 7379 (6049) |

Fig.14 Dose response curves of u.v. induced DNA repair in lymphocytes derived from AK patients and age-matched normal individuals measured by the total incorporation method. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3) were irra ranging from 2.5 to 20 $J.m^{-2}$ or mock irradiated and rang incubated immediately with $[^3H]$- dT (5 $\mu$Ci/ml, 18.5Ci/mmole) incul and OH-urea ($1.5 \times 10^{-3}$M) at 37°C for 4 h. After and OH-urea ($1.5 \times 10^{-3}$M) at 37°C for 4 h. After incubation, the cultures were washed, fixed and assayed for $[^3H]$- dT incorporation as described in methods section 2.1.6. Each value was obtained by subtracting from the d.p.m. for each irradiated culture, the mean of d.p.m. for duplicate unirradiated controls of the same donor. Points represent the means of duplicate determinations in different cultures for each dose and bars represent the ranges. The counting efficiency was determined by the channels ratio method.

○ Normal lymphocytes

● AK lymphocytes


[3H] - dT incorporation into acid insoluble material
(d p m x 10-3/10^6 cells)
1
2
3
4
5
6
25
100
200
u.v. dose (J.m-2)

in unirradiated cells is a significant proportion of that in irradiated cells undergoing repair (e.g. 30 - 40% of that at 10 $J.m^{-2}$). However, for the purpose of calculating DNA repair, the inhibition of DNA replication (which is the same in both cell types, Results Section 3.11) is ignored.

The most interesting features shown by these results are:-

1) The $[^3H]$ - dT incorporation into unirradiated AK and normal lymphocytes is inhibited to the same extent by OH-urea.
2) The $[^3H]$ - dT incorporation increases after u.v. irradiation and the dose response is very similar to that obtained by autoradiographic analysis (see Fig. 9).
3) The difference in u.v. induced $[^3H]$ - dT incorporation in the presence of OH-urea, between AK and normal lymphocytes is independent of u.v. dose and is in a very good agreement with that obtained by autoradiographic analysis.

It should be noted that the values for u.v. induced $[^3H]$ - dT incorporation shown in Table 9 are minimum values. U.v. irradiation inhibits DNA replication (Painter et al 1970), so the contribution of replication to the total incorporation of $[^3H]$ - dT into irradiated cells must be less than the incorporation measured in unirradiated cells. However, as shown later, this inhibition of DNA replication is quantitatively similar in normal and AK lymphocytes (Results Section 3.11) and can thus be ignored.

In order to confirm that the reduced rate of u.v. induced $[^3H]$ - dT incorporation (DNA repair) found in the preliminary experiments (sections 3.3 & 3.4) and to extend the autoradiographic analysis (section 3.6) to show that it is a general characteristic of the AK condition, a larger number of AK patients, covering conditions showing different degrees of severity (as diagnosed by the clinician) need to be tested, by the much easier and faster total incorporation method.

Some difficulties were encountered during the course of this work. Although AK is a condition known to be common in the West of Scotland, there were periods when cases were not available and the samples of blood collected on these occasions when patients could be found varied in volume from 4 ml to the preferred sample size of 20 ml. When an adequate sample was provided, the estimations (which were always carried out in duplicate) were performed with two different radiation doses and the $[^3H]$ - dT incorporation of the lymphocytes in the absence of OH-urea was also measured. When the blood sample was small ( 10 ml), the estimations were only performed at one radiation dose and when the sample was very small ( 6 ml), the control with OH-urea was omitted (see Tables 10 & 11).

Tables 10 & 11 show results of an analysis of DNA repair in lymphocytes derived from seventeen AK patients and the same number of age-matched normal individuals, irradiated at 10 and 20 $J.m^{-2}$. The reproducibility of the data obtained by this method is good and in all determinations the u.v. induced $[^3H]$ - dT incorporation is lower in AK lymphocytes than in normal lymphocytes.

Table 10 Estimation of u.v. induced DNA repair and the % inhibition of DNA replication with OH-urea in lymphocytes derived from AK patients and age-matched normal individuals, by the total indorporation method. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3), were incubated immediately after irradiation (10.0 $J.m^{-2}$) or mock irradiation with $[^3H]$ - dT (5 /uCi/ml, 21 Ci/mmole) and OH-urea ($1.5 \times 10^{-3}$M) at 37°C for 4 h. For the determination of % inhibition of DNA replication by OH-urea, unirradiated cultures were incubated in the absence of the drug under exactly the same conditions. After incubation, the cultures were washed, fixed and assayed for $[^3H]$ - dT incorporation as described in methods section 2.1.6. The counting efficiency was determined by the channels ratio method. The u.v. induced DNA repair values were obtained by subtracting the mean d p m of duplicate unirradiated cultures from the d p m of the corresponding irradiated culture. The % inhibition of DNA replication by OH-urea was calculated as

$$100 \times \frac{\left(\begin{matrix}\text{mean dpm of duplicate} \\ \text{unirr. cultures - OH urea}\end{matrix}\right) - \left(\begin{matrix}\text{mean dpm of duplicate} \\ \text{unirr. cultures + OH urea}\end{matrix}\right)}{\left(\begin{matrix}\text{mean dpm of duplicate unirr. cultures - OH urea}\end{matrix}\right)}$$

ND = Not determined

| Blood type | Total $[^3H]$ - dT (dpm) incorporated into $10^6$ unirradiated cells in the presence of OH-urea | | DNA repair measured as the difference in $[^3H]$ - dT (dpm) incorporated per $10^6$ cells between irradiated (10.0 $J.m^{-2}$) and unirradiated cells from the same donor | | % DNA replication inhibited by OH-urea |
|---|---|---|---|---|---|
| | Duplicate 1 | Duplicate 2 | Duplicate 1 | Duplicate 2 | |
| AK 15 | 1239 | 1415 | 1693 | 1868 | 75.5 |
| NL 15 | 1356 | 1429 | 4635 | 4854 | 78.5 |
| AK 16 | 1521 | 1336 | 1644 | 1694 | 60.8 |
| NL 16 | 1423 | 1287 | 4340 | 4100 | 74.6 |
| AK 17 | 1610 | 1450 | 2300 | 2206 | N.D |
| NL 17 | 1553 | 1349 | 5389 | 5321 | N.D |
| AK 18 | 1408 | 1382 | 2488 | 2381 | 84.9 |
| NL 18 | 1205 | 1340 | 6055 | 6176 | 81.0 |
| AK 19 | 1358 | 1290 | 2183 | 2077 | 78.8 |
| NL 19 | 1442 | 1536 | 4973 | 5135 | 71.0 |
| AK 20 | 1040 | 1420 | 2640 | 2476 | N.D |
| NL 20 | 1576 | 1489 | 4421 | 4825 | 70.4 |
| AK 21 | 1214 | 1430 | 2280 | 2480 | 75.1 |
| NL 21 | 1318 | 1384 | 5282 | 5040 | 79.0 |

Table 11 Estimation of u.v. induced DNA repair and the % inhibition of DNA replication with OH-urea in lymphocytes derived from AK patients and age-matched normal individuals, by the total incorporation method. The experimental details and working out the results are the same as described in the legend to table 10, except that cultures were irradiated at 20 $J.m^{-2}$ instead of 10 $J.m^{-2}$.

ND = Not determined

| Blood type | Total $[^3H]$ - dT (dpm) incorporated into $10^6$ unirradiated cells in the presence of OH-urea | | DNA repair measured as the difference in $[^3H]$ - dT (dpm) incorporated per $10^6$ cells between irridiated (20 J.m$^{-2}$) and unirridiated cells from the same donor | | % DNA replication inhibited by OH-urea |
|---|---|---|---|---|---|
| | Duplicate 1 | Duplicate 2 | Duplicate 1 | Duplicate 2 | |
| AK 22 | 1448 | 1251 | 2380 | 2673 | N.D |
| NL 22 | 1534 | 1318 | 4650 | 4376 | N.D |
| AK 23 | 1214 | | 2703 | 2623 | 79.0 |
| NL 23 | 1360 | 1410 | 5850 | 5530 | 74.0 |
| AK 24 | 1810 | 1560 | 2193 | 2086 | N.D |
| NL 24 | 1444 | 1615 | 5073 | 4893 | N.D |
| AK 25 | 1486 | 1393 | 1886 | 1768 | 70.5 |
| NL 25 | 1512 | 1216 | 4720 | 4450 | 69.0 |
| AK 26 | 1335 | | 3516 | 3483 | 86.0 |
| NL 26 | 1219 | | 5446 | 5573 | 80.5 |
| AK 27 | 1414 | | 2360 | 2206 | N.D |
| NL 27 | 1503 | 1309 | 5436 | 5230 | 73.0 |
| AK 28 | 1834 | 1567 | 2730 | 2520 | 75.0 |
| NL 28 | 1712 | 1629 | 6130 | 5948 | 78.6 |
| AK 29 | 1322 | 1348 | 1943 | 1913 | 68.3 |
| NL 29 | 1417 | | 4740 | 5020 | 72.4 |
| AK 30 | 1341 | 1366 | 2013 | 2116 | N.D |
| NL 30 | 1272 | 1317 | 5046 | 4950 | N.D |

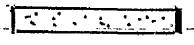
Figs 15 & 16 show the same data in the form of histograms which show more clearly the range of variation in both the AK and normal cell types. Except for one value (Fig. 16) where u.v. induced $[^3H]$ - dT incorporation in AK is 63% that of normal, the rest of the values for AK cells all fall between 38 - 52% of the corresponding values for the normal cells. The variation in the rate of DNA repair for AK patients may be due to the degree of severity of the condition as is found with Xeroderma Pigmentosum (see Section 1.5).

It is reasonable to assume, like other authors (Burk et al 1971a; Robbins and Kraemer 1972a & b; Lambert et al 1976), that the u.v. induced $[^3H]$ - dT estimated by the total incorporation method, is a measure of DNA repair. However, in this work the assumption has been tested and shown to be correct (see Results Section 3.8). Furthermore, the observed difference in $[^3H]$ - dT incorporation between AK and normal lymphocytes is not necessarily caused by a difference in the rate of DNA repair. Once again, (as already discussed in Results Section 3.6) differences in the rate of dT incorporation into the nucleotide pools and the pool sizes themselves may vary from one cell type to another and thus influence the rate of $[^3H]$ - dT incorporation.

Fig. 15 Histogram of the different values of u.v. induced DNA repair presented in table 10, showing the difference between AK patients and age-matched normal individuals and also the range of variation in each cell type. The experimental details are given in the legend to table 10.

Fig. 16 Histogram of the different values of u.v. induced DNA repair presented in table 11, showing the difference between AK patients and age-matched normal individuals and also the range of variation in each cell type. The experimental details are given in the legend to table 11.


Repair in AK patients


Repair in normal patients

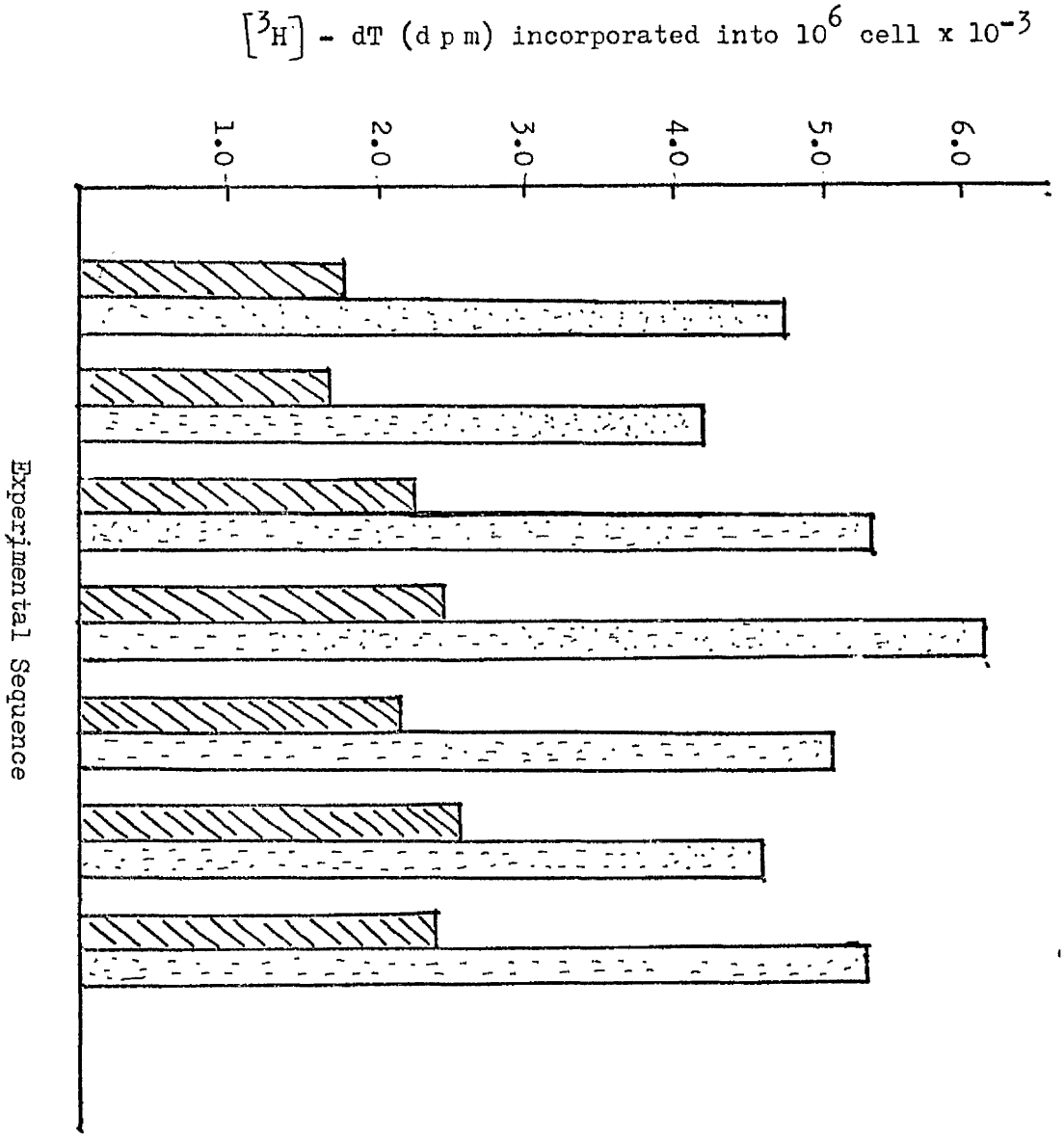

$[^3H]$ - dT (d p m) incorporated into $10^6$ cell x $10^{-3}$
1.0
2.0
3.0
4.0
5.0
6.0
Experimental Sequence

Fig. 15

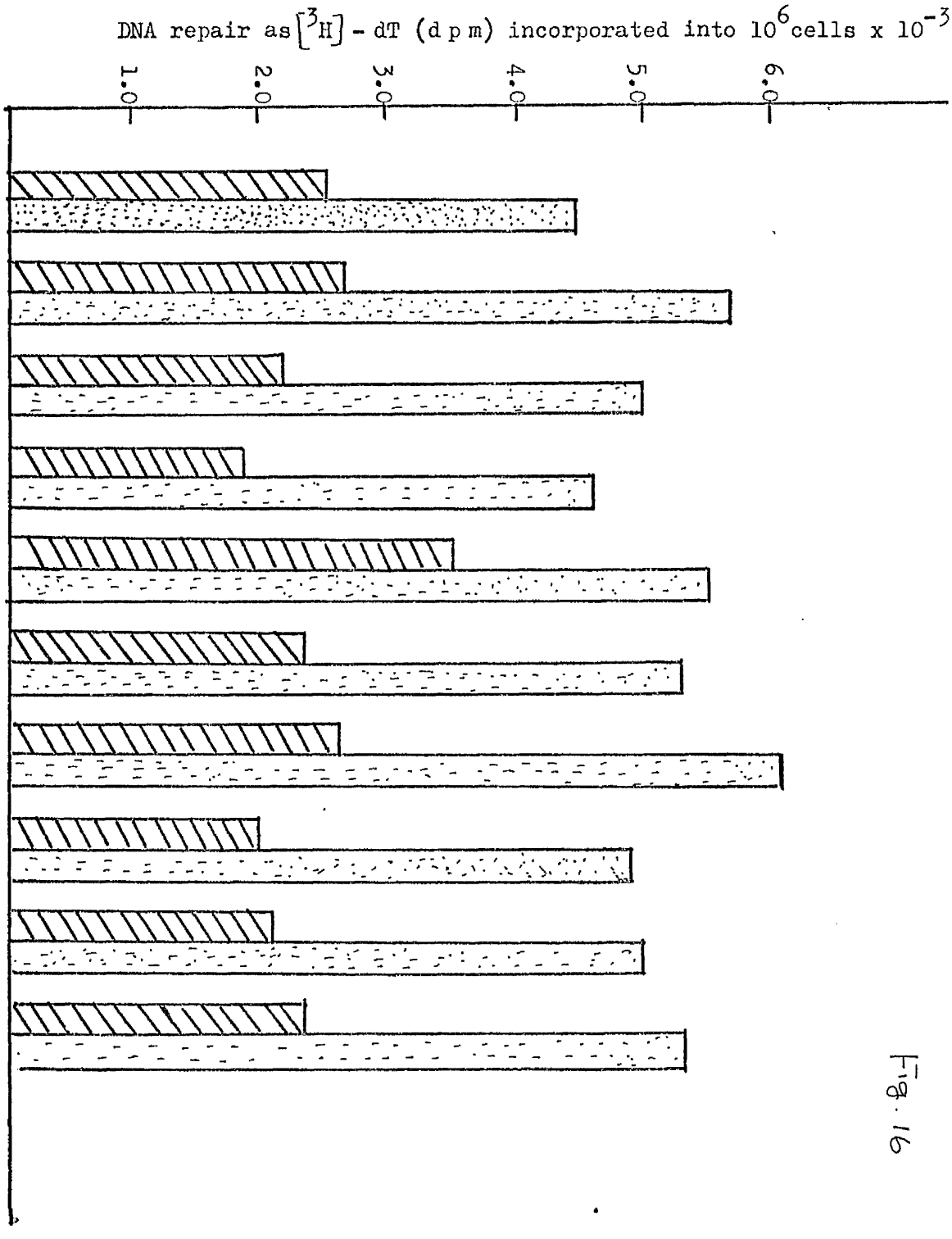

DNA repair as [3H] - dT (d p m) incorporated into 10^6 cells x 10^-3
1.0
2.0
3.0
4.0
5.0
6.0
Experimental Sequence

Fig. 16

3.8 Characterisation of u.v. induced DNA synthesis as DNA repair

The total incorporation of [$^3$H] - dT into OH-urea treated lymphocytes increases after the cells have been irradiated with u.v. light. It seems very likely that this u.v. induced incorporation represents DNA repair synthesis but it could also be due to some unusual form of induced replication (e.g. induction of bacteriophage replication by u.v. irradiation of lysogenic bacteria, Lwoff et al 1950). However, DNA replication and DNA repair can be distinguished by following DNA synthesis with the labelled thymidine analogue [$^3$H]- bromodeoxyuridine ([$^3$H]- BrdUrd).

In such experiments [$^3$H] - BrdUrd is substituted for [$^3$H] - dT as a DNA precursor. The incorporation of BrdUrd into replicating DNA leads to an increase in the buoyant density of the newly synthesized DNA. In DNA undergoing repair however, only small stretches on DNA are replaced by repair synthesized and hence very little or no change in buoyant density is observed (Hanawalt et al 1971). These two types of DNA synthesis can therefore be distinguished by density gradient centrifugation.

CsCl density gradient centrifugation profiles of [$^3$H] - BrdUrd labelled DNA from unirradiated and irradiated OH-urea treated lymphocytes derived from normal individuals are shown in Fig. 17. The DNA from the unirradiated lymphocytes forms only one peak of radioactivity which is observed in the hybrid density region. The peak is clearly separated from the bulk unlabelled DNA (see 260 nm extinction profiles in Fig. 17a). This result is expected and represents the residual DNA replication

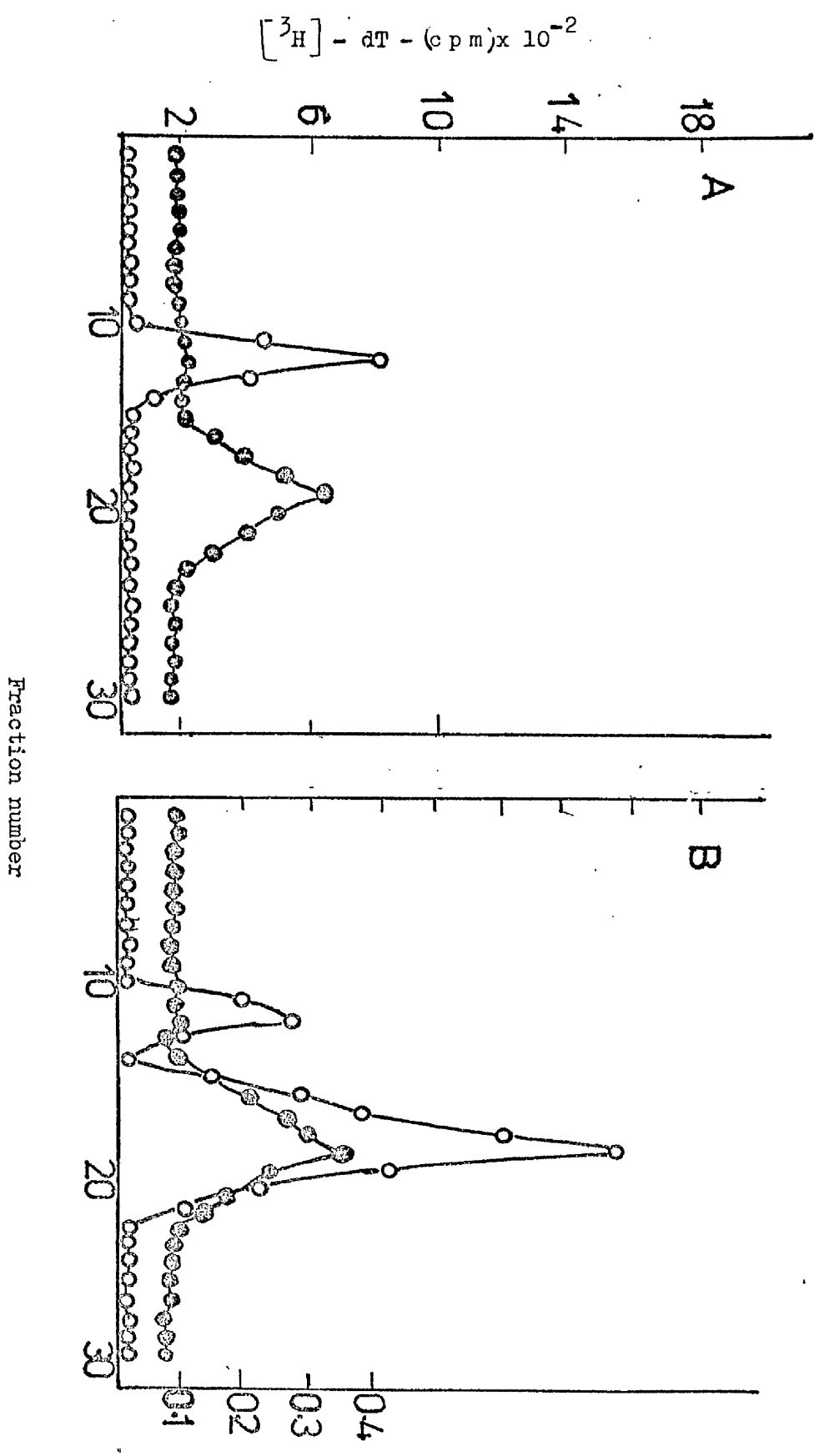
Fig. 17 Caesium chloride density gradients centrifugation profiles of DNA obtained with lymphocytes derived from normal subjects. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3) were irradiated at 20 $J.m^{-2}$ or mock irradiated and incubated immediately with [$^3$H] - BrdUrd (10 $\mu$Ci/ml, 2.2 Ci/mmole), FdUrd ($10^{-6}$ M) and OH-urea ($1.5 \times 10^{-3}$ M) at 37°C for 4 h. After incubation, the content of two identical cultures was pooled, and cells were washed and suspended in 3.0 ml SDS in saline-citrate buffer pH 7.0 (0.1% w/v). DNA was isolated and centrifuged as described in methods section 2.1.8. 30 fractions were collected and the radioactivity and extinction at 260 nm for each fraction were measured as described in methods section 2.1.8. The counting efficiency was determined by the channels ratio method.

A non irradiated cells

B irradiated 20 $J.m^{-2}$

○ [$^3$H] - BrdUrd (cpm)

● Extinction at 260 nm


[3H] - dT - (c p m)x 10-2
2
6
10
14
18
A
10
20
30
B
10
20
30
0.1
0.2
0.3
0.4
Fraction number
Extinction at 260 nm

not inhibited by OH-urea in the small percentage (<1%) of cells in S-phase.

After irradiation of the lymphocytes with a dose of 20 $J.m^{-2}$ however, the labelled DNA forms two distinct peaks (Fig. 17b), the larger peak, which coincided exactly with the bulk unlabelled DNA in the normal density region, represents DNA repair. The smaller peak is in the same position as that observed in unirradiated lymphocytes and represents DNA replication.

The % inhibition of DNA replication by u.v. light (20 $J.m^{-2}$) can be calculated from Fig. 17 by measuring the decrease in the activity of the DNA in the hybrid density region due to u.v.-irradiation. This value (28%) is reasonably close to the u.v. inhibition (25%, see Fig. 27) of DNA replication in PHA stimulated lymphocytes (see Results Section 3.11).

3.9 Determination of endogenous thymidine nucleotide pool sizes and the true rates of DNA synthesis in normal and AK lymphocytes

Almost all reports on the u.v. stimulated incorporation of $[^3H]$ - dT into mammalian cells, implicitly assume that any apparent difference in the uptake of label found between 2 cell types is necessarily due to a difference in their rate of DNA synthesis.

Since the extent of dilution of $[^3H]$ - dT nucleotides derived from added $[^3H]$ - dT depends on the relative rates of endogenous thymidine nucleotide synthesis and the synthesis of thymidine nucleotide from exogenous thymidine in the medium,

$[^3H]$ - dT incorporation into DNA will depend on the relative activities of the endogenous pathway (thymidylate synthetase), and the exogenous pathway (thymidine transport and thymidine kinase). If u.v. stimulated DNA repair in two cell types is compared, the authenticity of any apparent difference in the rate of DNA synthesis may be challenged, unless the endogenous thymidine nucleotide pool sizes have been shown to be similar in each cell type.

In order to overcome this type of criticism of the conclusions drawn in this study of u.v. induced DNA repair in lymphocytes derived from AK patients and age-matched normal individuals, the thymidine nucleotides pool sizes and the true rates of DNA synthesis for each cell type were measured, using the method described by Adams (1969). This determination is based on the assumptions that the rate of DNA synthesis is independent of the exogenous dT concentration and the thymidine nucleotide pool sizes and that the radioactivity incorporated into DNA in a given time depends only on the specific activity of the $[^3H]$- dTTP precursor. It is also assumed that the acid-soluble thymidine nucleotide pools are mainly in the form of $[^3H]$ - dTTP (Munch-Petersen et al 1973).

From these assumptions, the following equation was developed by Adams (1969), thus

$$\frac{[^3H]\text{- dTTP}}{[^3H]\text{- dTTP} + \text{dTTP}} = \frac{[^3H]\text{- DNA}}{\text{DNA}(M)} \qquad \ldots\ldots (1)$$

where DNA(M) is the maximum incorporation of radioactivity into

DNA (and a measure of the true rate of DNA synthesis) which would be found if the $[^3H]$ - dTTP precursor had the same specific activity as $[^3H]$ - dT supplied in the medium i.e. $[^3H]$- dTTP is very large relative to dTTP . $[^3H]$- DNA is the observed incorporation of $[^3H]$ - dT into DNA, and dTTP and $[^3H]$- dTTP are the concentrations of the endogenously derived and exogenously derived thymidine triphosphate pools respectively.

Rearranging equation (1), thus

$$\frac{1}{[^3H]\text{- DNA}} = \frac{dTTP}{DNA(M)} \times \frac{1}{[^3H]\text{- dTTP}} + \frac{1}{DNA(M)}$$

when $\frac{1}{[^3H]\text{ - DNA}}$ is plotted against $\frac{1}{[^3H]\text{- dTTP}}$, this equation represents a straight line of

$$\text{slope} = \frac{dTTP}{DNA(M)} \quad \text{and intercept} = \frac{1}{DNA(M)}$$

By measuring the incorporation of $[^3H]$ - dT into acid-soluble nucleotide and acid-insoluble (DNA) fractions of cells at various exogenous $[^3H]$ - dT concentrations, the true rate of DNA synthesis can be obtained by extrapolation to $\frac{1}{[^3H]\text{- dTTP}} = 0$ when the dTTP pool would be entirely derived from the exogenous $[^3H]$ - dT and dTTP can be calculated.

It is important that $[^3H]$- dTTP and dTTP are of the same order of magnitude otherwise the analysis does not give useful results. The incubation of u.v. irradiated lymphocytes with $[^3H]$ - dT

varying in concentration from $2.5 \times 10^{-7}$ M (which is the concentration of the labelled thymidine in the absence of cold thymidine) to $4.0 \times 10^{-6}$ M, was found to be suitable (see Figs 18 & 19). This range covers more than a tenfold difference in the concentration of $[^3H]$ - dT.

However, the incorporation of $[^3H]$ - dT into the acid-soluble pool (and hence $[^3H]$- dTTP ) increases with time until a steady state situation is reached when the rate of synthesis is equal to the rate of incorporation into DNA. In human lymphocyte system this steady state is reached $\frac{1}{2}$ h after the addition of $[^3H]$ - dT to the cells as shown in Fig. 20.

Incorporation of $[^3H]$ - dT into acid-soluble and acid-insoluble fractions of $3.0 \times 10^6$ u.v. irradiated lymphocytes was measured after incubation with different exogenous $[^3H]$ - dT concentrations, in the presence of OH-urea for 4 h (i.e. under the conditions of the DNA repair assay). The data are plotted as incorporation into acid-insoluble (DNA, Fig. 18) or acid-soluble (nucleotide pool, Fig. 19) fractions (estimated as described in methods section 3.1.9) against exogenous $[^3H]$ - dT concentration. The results plotted this way, suggest that the rate of DNA synthesis is different in AK and normal lymphocytes but that the dT - nucleotide pool sizes are similar because there is a constant ratio between the values for incorporation into DNA of the two cell types while the incorporation into the nucleotide pools are very similar at all the different exogenous $[^3H]$ - dT concentrations.

Fig. 18 The effect of different concentrations of $[^3H]$ - dT on the rate of incorporation of radioactivity into DNA of u.v. irradiated lymphocytes derived from AK patients and age-matched normal individuals. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3) were incubated immediately after u.v. irradiation (20 $J.m^{-2}$) with different concentration of $[^3H]$ - dT varying from $2.5 \times 10^{-7}$M to $4.0 \times 10^{-6}$M and OH-urea ($1.5 \times 10^{-3}$M) at 37°C for 4 h. After incubation, the cultures were washed and assayed for $[^3H]$ - dT incorporation into DNA as described in methods section 2.1.9. The efficiency of counting was determined by the channels ratio method.

○ Normal, ● AK

Fig. 19 The effect of different concentrations of $[^3H]$ - dT on the incorporation of radioactivity into acid soluble fractions of u.v. irradiated lymphocytes derived from AK patients and age-matched normal individuals. Cultures of $3 \times 10^6$ cells were treated in exactly the same as described in Fig. 18. After incubation, the cultures were washed and assayed for $[^3H]$ - dT incorporation into the acid-soluble fractions as described in methods section 2.1.9. The efficiency of counting was determined by the channels ratio method.

○ Normal, ● AK

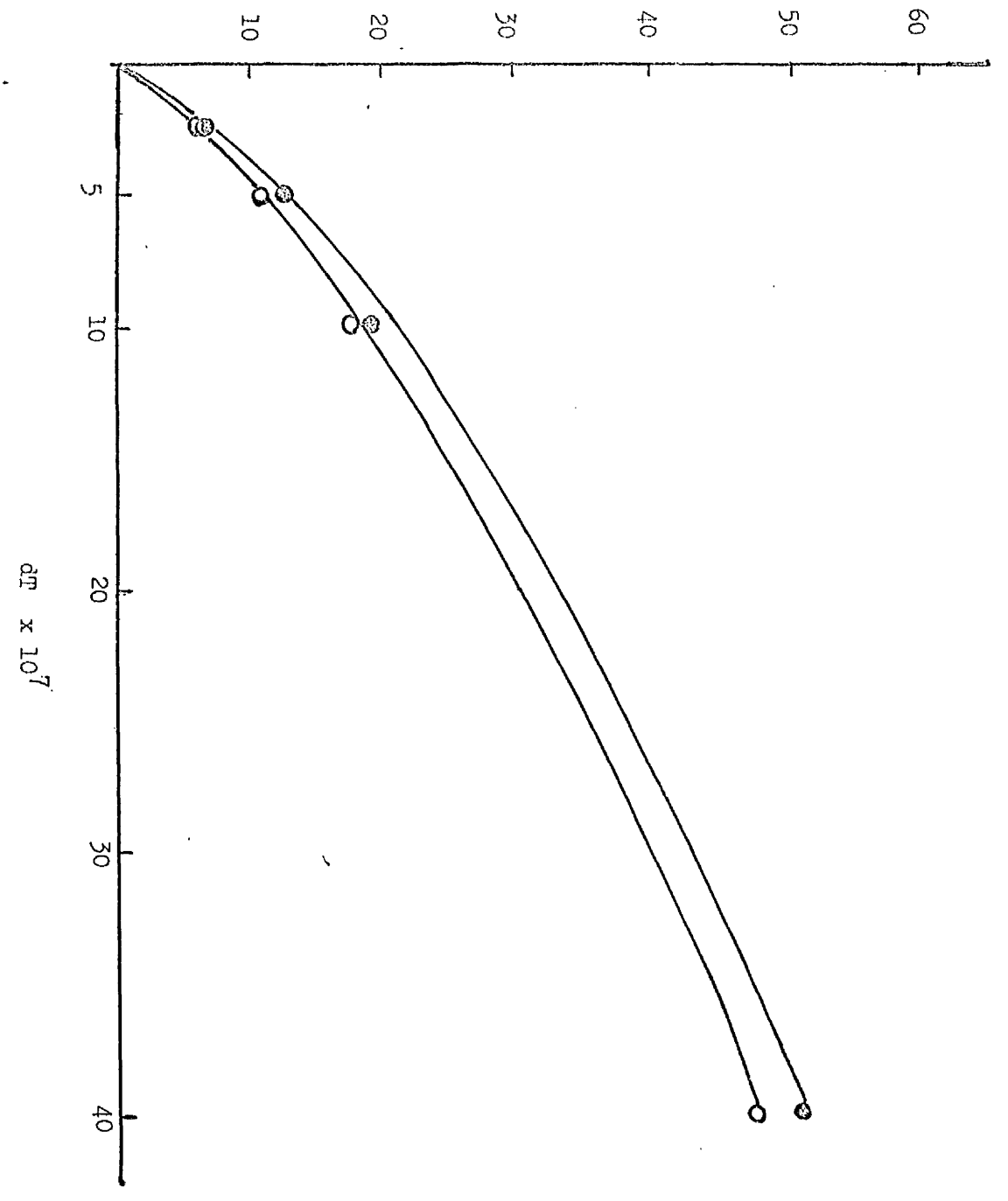

$10^{-3}$ x total [$^3$H]- dT (d p m) incorporated into A S per culture
10
20
30
40
50
60
5
10
20
30
40
dT x $10^7$

Fig. 19

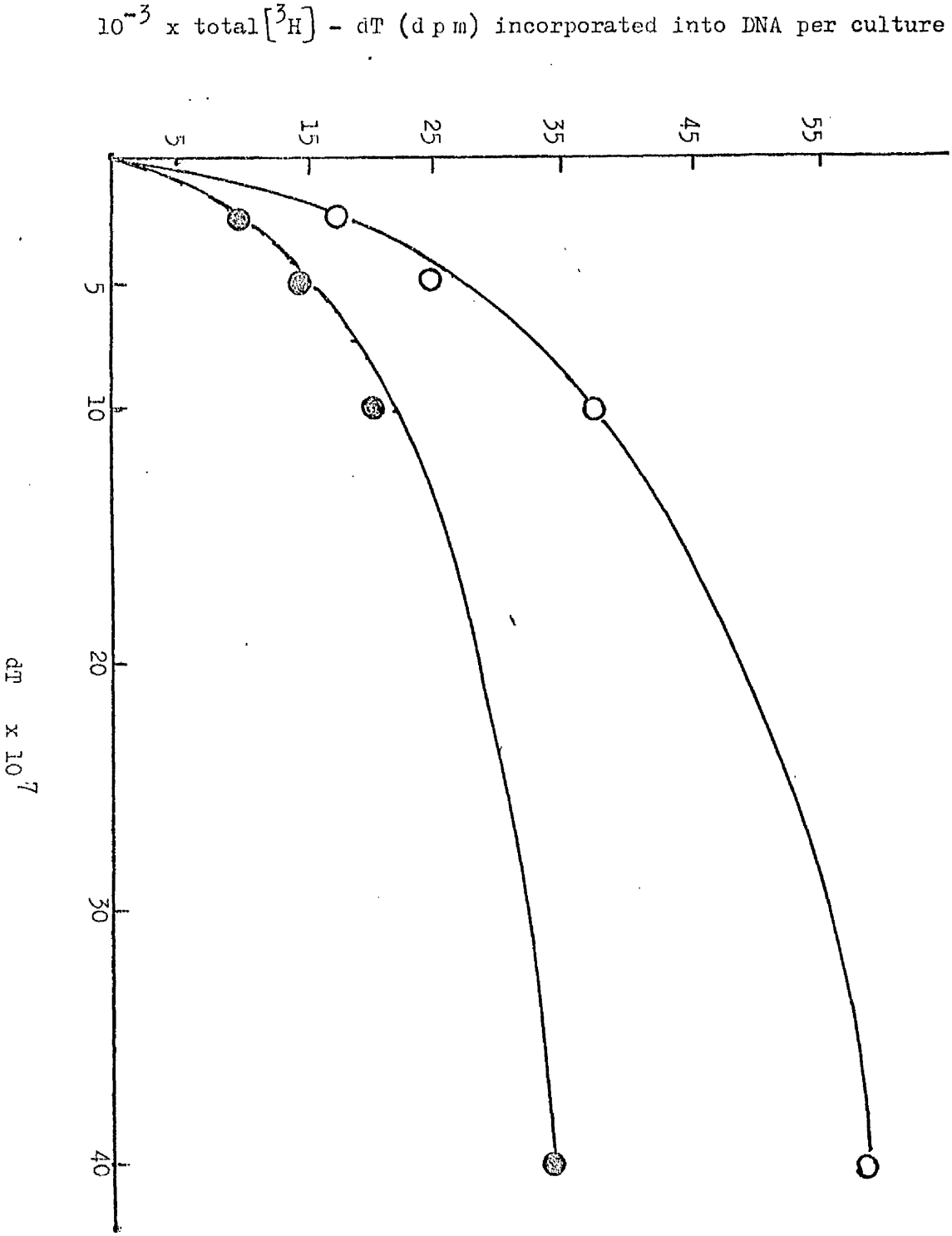

$10^{-3}$ x total [$^3$H] - dT (d p m) incorporated into DNA per culture
5
15
25
35
45
55
5
10
20
30
40
dT x $10^{-7}$

Fig. 18

Fig. 20 Time course of $[^3H]$ - dT incorporation into acid-soluble pools of lymphocytes derived from a normal subject. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3), were incubated immediately after irradiation (20 $J.m^{-2}$) with $^3H$ - dT (5 $\mu$Ci/ml, 21 Ci/mmole) and Ol-urea ($1.5 \times 10^{-3}$M) for different times from 0 to 4 h at 37°C. After incubation, the cultures were washed and assayed for $[^3H]$ - dT incorporation into the acid-soluble fractions as described in methods section 2.1.9. The efficiency of counting was determined by the channels ratio method.

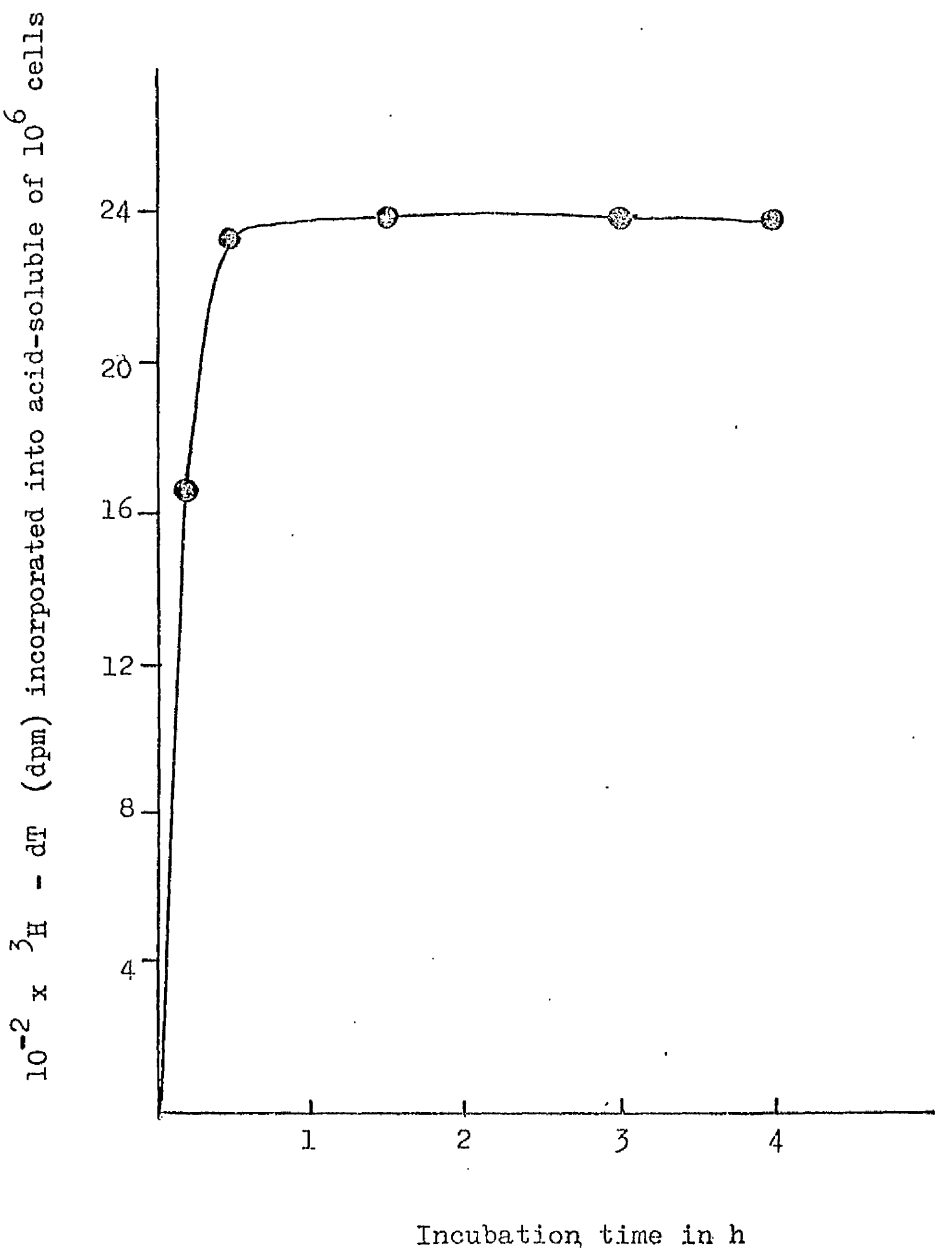

$10^{-2}$ x $^{3}H$ - dT (dpm) incorporated into acid-soluble of $10^{6}$ cells
24
20
16
12
8
4
1
2
3
4
Incubation time in h

Fig. 21 is a double reciprocal plot using the same data of the radioactivity incorporated into DNA against that incorporated into the nucleotide pool, from which the true rate of DNA synthesis and the endogenous dTTP for both AK and normal lymphocytes can be obtained.

The straight lines shown on the graph (Fig. 21) were drawn through 2 points, the coordinates of which were determined by calculating the slope of the line using the least square method for best fit. All 4 points representing each straight line were given equal weighting.

Since we are interested in comparing the rate of DNA synthesis and the endogenous dTTP pool size between AK and normal lymphocytes, no attempt was made to work out their respective absolute values.

The two straight lines when extrapolated cut the Y-axis at different points (Fig. 21) showing the true rate of u.v. induced DNA repair synthesis in both cell types (i.e. the rate of $[^3H]$ - dT incorporation into DNA at an infinitely high exogenous $[^3H]$ - dT concentration, when $[^3H]$- dTTP is very high compared to dTTP ) is different in the two cell types. The rate in AK lymphocytes from this data is 52% of that in normal lymphocytes. In three determinations in different experiments with different samples of

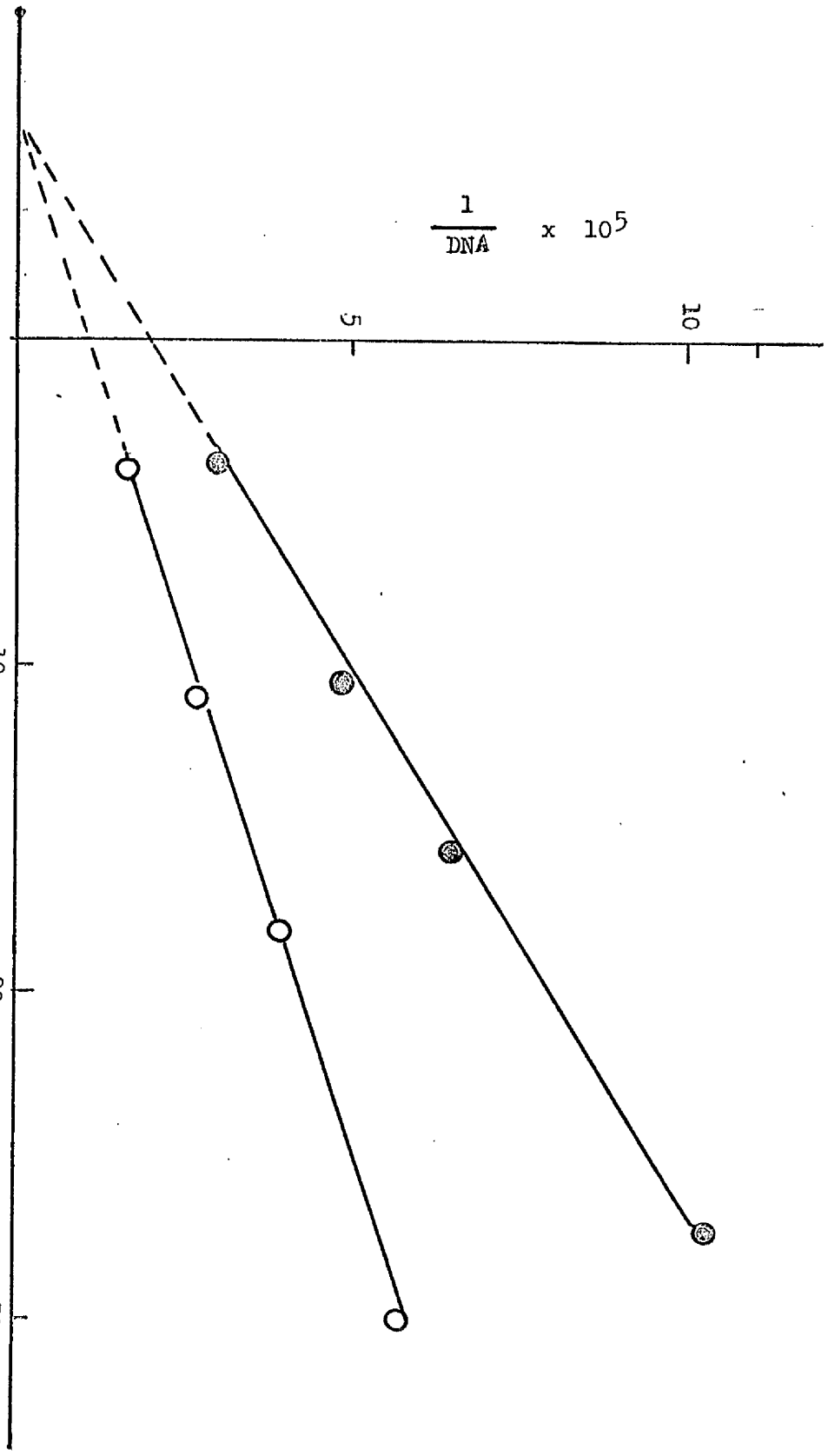
Fig. 21 Estimation of endogenous pools of dTTP and true rates of DNA synthesis in lymphocytes derived from AK patients and age-matched normal individuals. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3) were incubated immediately after irradiation (20 $J.m^{-2}$) with [$^3$H] - dT at concentrations ranging from $2.5 \times 10^{-7}$M to $4.0 \times 10^{-6}$M and OH-urea ($1.5 \times 10^{-3}$M) at 37°C for 4 h. After incubation, the cultures were washed and the incorporation of radioactivity into acid soluble (AS) and into DNA was measured as described in methods section 2.1.9. Each straight line was drawn through 2 points, the coordinates of which were determined by calculating the slope of the line using the least square method. The lines were extended and their intercepts on both the Y and X axis were measured. The counting efficiency was determined by the channels ratio method.

Ordinate represents $\frac{1}{[^3H]\text{- DNA}}$

Abscissa represents $\frac{1}{AS}$

● AK

○ Normal


$\frac{1}{DNA} \times 10^5$
5
10
10
20
30
$\frac{1}{A_S} \times 10^5$

AK and age-matched control lymphocytes, the true rate of DNA repair synthesis in AK cells was 49 ± 3% that in normal cells.

These results substantiate the earlier conclusions which suggested that the rate of DNA repair in AK lymphocytes is 4 that of normal lymphocytes and constitute good evidence that the difference in [$^3$H] - dT incorporation represents different rates of DNA synthesis in the two cell types.

3.10 Analysis of prolonged DNA repair in AK lymphocytes

The preliminary experiments on the time course of DNA repair (see Fig. 8) suggested that while u.v. stimulated DNA repair in normal lymphocytes has reached a plateau 4 h after irradiation, repair in AK lymphocytes at this time is still in progress. This is interesting in view of the reports by Burk et al (1971a) and Robbins and Kraemer (1972a & b) which demonstrated that XP lymphocytes have a decreased rate of u.v. induced [$^3$H] - dT incorporation immediately after irradiation but continue to incorporate the label for a longer time than normal lymphocytes.

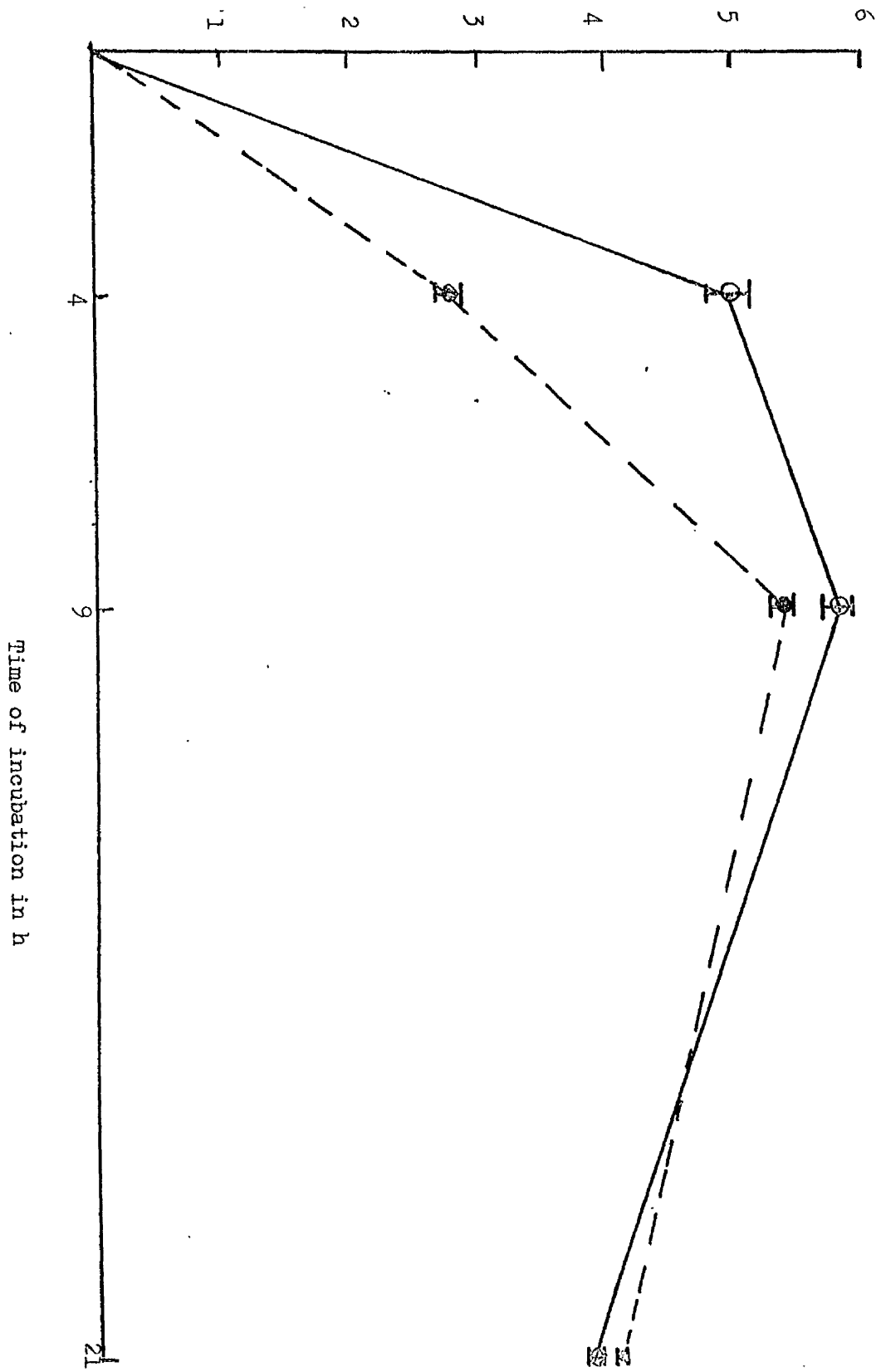
Since AK resembles in some ways, the less severe forms of XP in that both are preneoplastic conditions (Introduction Sections 1.6 & 1.5) and have reduced rates of DNA repair (Results Sections 3.6 & 3.7), it seemed possible that AK lymphocytes although having a reduced rate of DNA repair, would complete the repair process if given sufficient time.

Fig. 22 shows the results of a prolonged analysis of DNA repair in AK lymphocytes, using the total incorporation method in

Fig. 22 Time course of prolonged u.v. stimulated DNA repair in lymphocytes derived from AK patients and age-matched normal individuals, measured by the total incorporation method. Cultures of $3 \times 10^6$ cells in a total volume of 4.0 ml medium (see methods section 2.1.3) were irradiated at 20 $J.m^{-2}$ or mock irradiated and incubated immediately with [$^3$H] - dT (5 $\mu$Ci/ml, 18.5 Ci/mmole) and OH-urea ($1.5 \times 10^{-3}$ M) at 37°C for different periods from 0 to 21 h. After incubation, the cultures were washed, fixed and assayed for [$^3$H] - dT incorporation as described in methods section 2.1.6. Each point was obtained by subtracting the mean d p m of duplicate unirradiated controls from the mean d p m of the corresponding irradiated cultures. The bars represent the ranges or repair values. The counting efficiency was determined by the channels ratio method.

○ Normal

● AK


$10^{-3}$ x DNA repair as $[^3H]$- dT incorporated (d p m) per $10^6$ cells
1
2
3
4
5
6
4
9
21
Time of incubation in h

the presence of OH-urea. The results clearly demonstrate that following u.v. irradiation (20 $J.m^{-2}$), AK lymphocytes eventually incorporate nearly as much $[^3H]$ - dT as do normal lymphocytes. The early part of the time cou$[^3H]$ - dT as do normal lymphocyte same as that obtained earlier (see Fig. 8) but while the normal cells do not incorporate much more $[^3H]$ - dT during the next 5 h (incorporation increases by 1% between 4 h and 9 h), the incorporation of label into AK cells continues to increase and almost doubles, almost reaching the same level (93%) as that into the normal cells. At even later time (21 h after irradiation) the total incorporation into AK cells is as great as that into normal cells.

The results presented by Robbins and Kraemer (1972a) showed that u.v. irradiated lymphocytes derived from patients with XP (common form) require 19 h to perform as much DNA repair as normal lymphocytes (C.f. AK lymphocytes require about 9 h for the same purpose). This is consistent with the slower rate of DNA repair shown to occur (Robbins and Kraemer 1972a) in XP lymphocytes (less than 30% that of normal lymphocytes after 4 h incubation, following u.v. irradiation) as compared to 50% that of the normal found in AK lymphocytes at the end of the same 4 h period.

## 3.11 U.v. induced DNA repair in PHA stimulated human lymphocytes

When human peripheral blood lymphocytes are incubated in standard culture media supplemented with 15% foetal calf serum, the cells remain viable for several days. However, without the addition of a stimulating agent (mitogen), fewer than 1% of the cells enter DNA synthesis during a 72 h incubation period (Epstein and Stohlman 1964). If the mitogen PHA, extracted from the kidney bean (Phaseolus vulgaris), is added to the culture, a series of changes is initiated. As a consequence DNA synthesis starts about 30 h after addition of the PHA (see Fig. 23) and reaches a peak 18 h later.

U.v. irradiation inhibits DNA replication (Cleaver 1967; Painter et al 1970), probably because thymine dimers block the progress of the growing points of newly synthesized daughter strands (Painter 1974). However, after removal of the dimers probably by the joint activities of the post-replication repair and excision repair systems (see Introduction Sections 1.4.3 and 1.4.4), DNA replication resumes.

The estimation of lymphocytes with PHA and the dependence of replication on repair have been combined to develop a new method of assessing DNA repair.

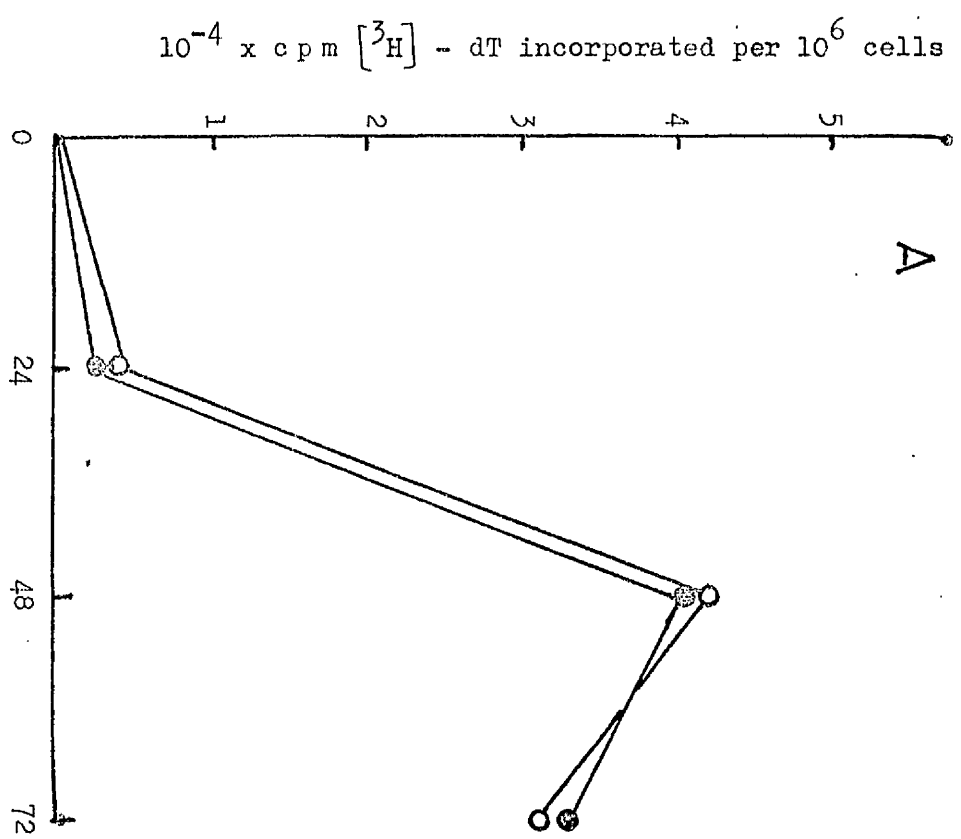
Lymphocytes were treated with PHA and later, near the peak of mitogen induced DNA synthesis (DNA replication), the cells were irradiated with u.v. light to inhibit $[^3H]$ - dT incorporation by 70 - 80%. Subsequent 1 h pulses of $[^3H]$ - dT were used to follow the

Fig. 23A Time course of $[^3H]$ - dT incorporation into PHA treated lymphocytes derived from AK patients and age-matched normal individuals. Setting up the cultures and the amount of PHA added were the same as described in the legend to Fig. 24. Cultures were incubated with PHA for different times (1 - 73 h) at 37°C and received an hourly pulse of $[^3H]$ - dT (0.5 $\mu$Ci/ml, 21 Ci/mmole) at the times shown. After incubation, the cultures were washed, fixed and assayed for $[^3H]$ - dT incorporation as described in methods section 2.1.6. The counting efficiency was determined by the channels ratio method.

Fig. 23B Estimation of the % cells incorporating $[^3H]$ - dT in the populations of lymphocytes described in Fig. 23A. Autoradiographs were prepared from the fixed suspensions of cells as described in methods section 2.1.5. For each determination, between 150 - 200 cells were counted and the number of cells incorporating the label was noted. The % of labelled cells was then estimated.

● AK patients lymphocytes

○ Normal patients lymphocytes


$10^{-4}$ x c p m [$^3$H] - dT incorporated per $10^6$ cells
0
1
2
3
4
5
24
48
72
A

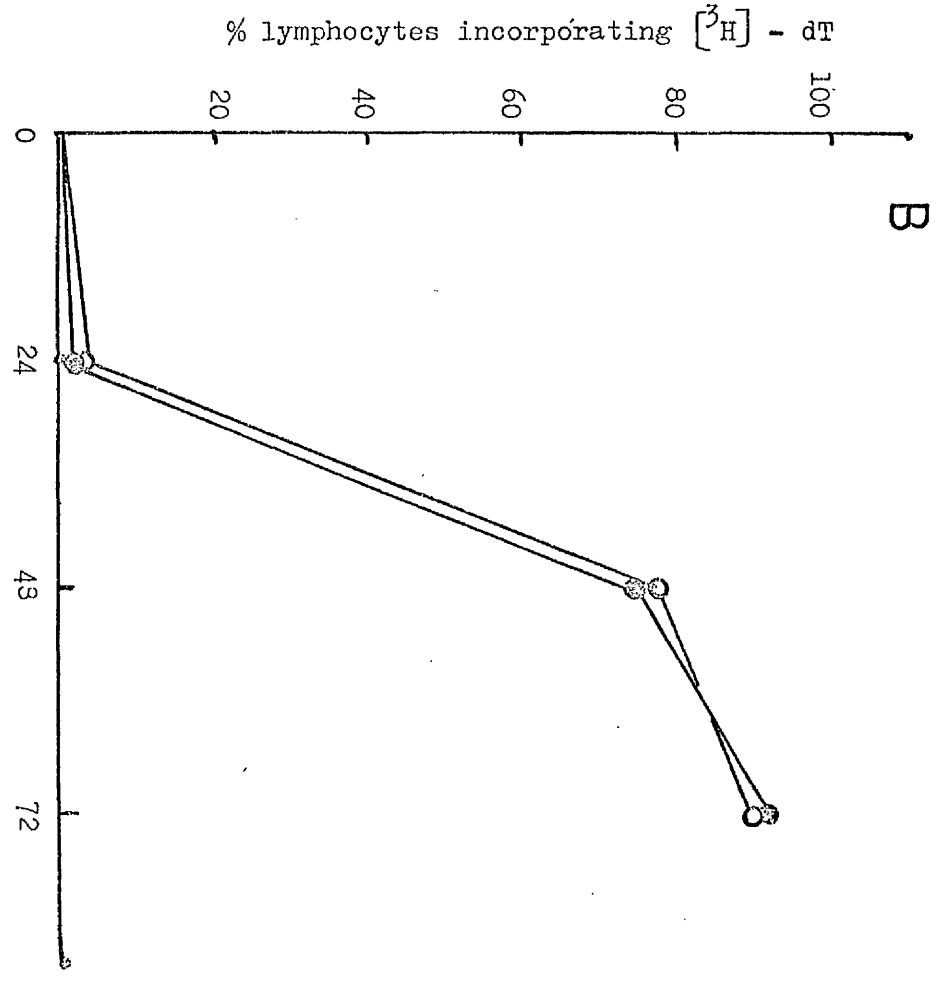
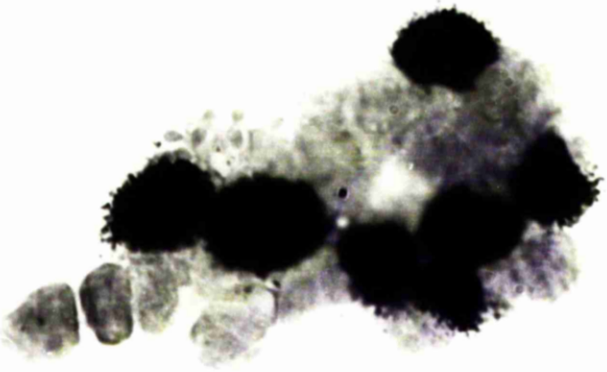

% lymphocytes incorporating [$^3$H] - dT
0
20
40
60
80
100
24
48
72
B

Plate 6 Autoradiographs of normal lymphocytes incubated with PHA for 48 h at 37°C, followed by 1 h pulse with [$^3$H] - dT. For experimental details see the legend to Fig. 23



recovery of DNA synthesis.

Fig. 24 shows the percentage inhibition of DNA synthesis 40 - 44 h after PHA stimulation in lymphocytes derived from three AK patients and three age-matched normal individuals after u.v. irradiation (100 $J.m^{-2}$ at 40 h after PHA treatment). The period of 40 h incubation of lymphocytes with PHA prior to u.v. irradiation was chosen, because it gave the largest difference in $[^3H]$ - dT incorporation between irradiated and unirradiated PHA stimulated lymphocytes (see Fig. 25). A period of 4 h seems to be sufficient time to allow the recovery of DNA replication in u.v. irradiated lymphocytes previously incubated with PHA for 40 h (see Fig. 26). It is clear from Fig. 26 that the repair of DNA in normal lymphocytes is almost complete 4 h after irradiation, while that in AK lymphocytes is only 50% that of unirradiated lymphocytes. This result confirms the time course of DNA repair for AK and normal lymphocytes established earlier (see Fig. 8) and also confirms the conclusion that the level of repair activity in AK cells is only half that in normal cells.

## 3.12 DNA repair in sun sensitive conditions other than AK

At the start of this work it was decided to examine one light sensitive condition in detail rather than make an extensive but less thorough survey of many different conditions. However, some preliminary data on DNA repair activity were obtained on cells from patients with lupus erythematosus (L.E.), basal cell carcinoma (B.C.C.) and melanoma (M).

Fig. 24 U.v. inhibition of S-phase DNA synthesis in PHA stimulated lymphocytes derived from 3 AK patients and the same number of age-matched normal individuals. Cultures of $10^6$ cells in a total volume of 2.0 ml medium (see methods section 2.1.3) were incubated with PHA (50 units) at 37°C for 40 h. The cultures were then removed from the incubator and lymphocytes isolated as described in methods section 2.1.7. Lymphocytes suspended in 1.0 ml PBS were placed in 50 mm petri dishes and irradiated at 100 $J.m^{-2}$ or mock irradiated. The original medium (2.0ml) containing PHA was added back to each culture together with 1.0 ml of RFCl5. Unirradiated cultures were treated in exactly the same way except for irradiation. All cultures were incubated at 37°C and received an hourly pulse of [$^3$H] - dT (0.50/$\mu$Ci/ml, 21 Ci/mmole) at the times shown. After incubation with the label, cultures were washed, fixed and assayed for [$^3$H] - dT incorporation as described in methods section 2.1.6. The datum is expressed as the percent reduction in the average c p m [$^3$H] - dT incorporated into duplicate irradiated cultures as compared to that of duplicate unirradiated controls. The counting efficiency was determined by the channels ratio method.

● AK patients lymphocytes

○ Normal subjects lymphocytes

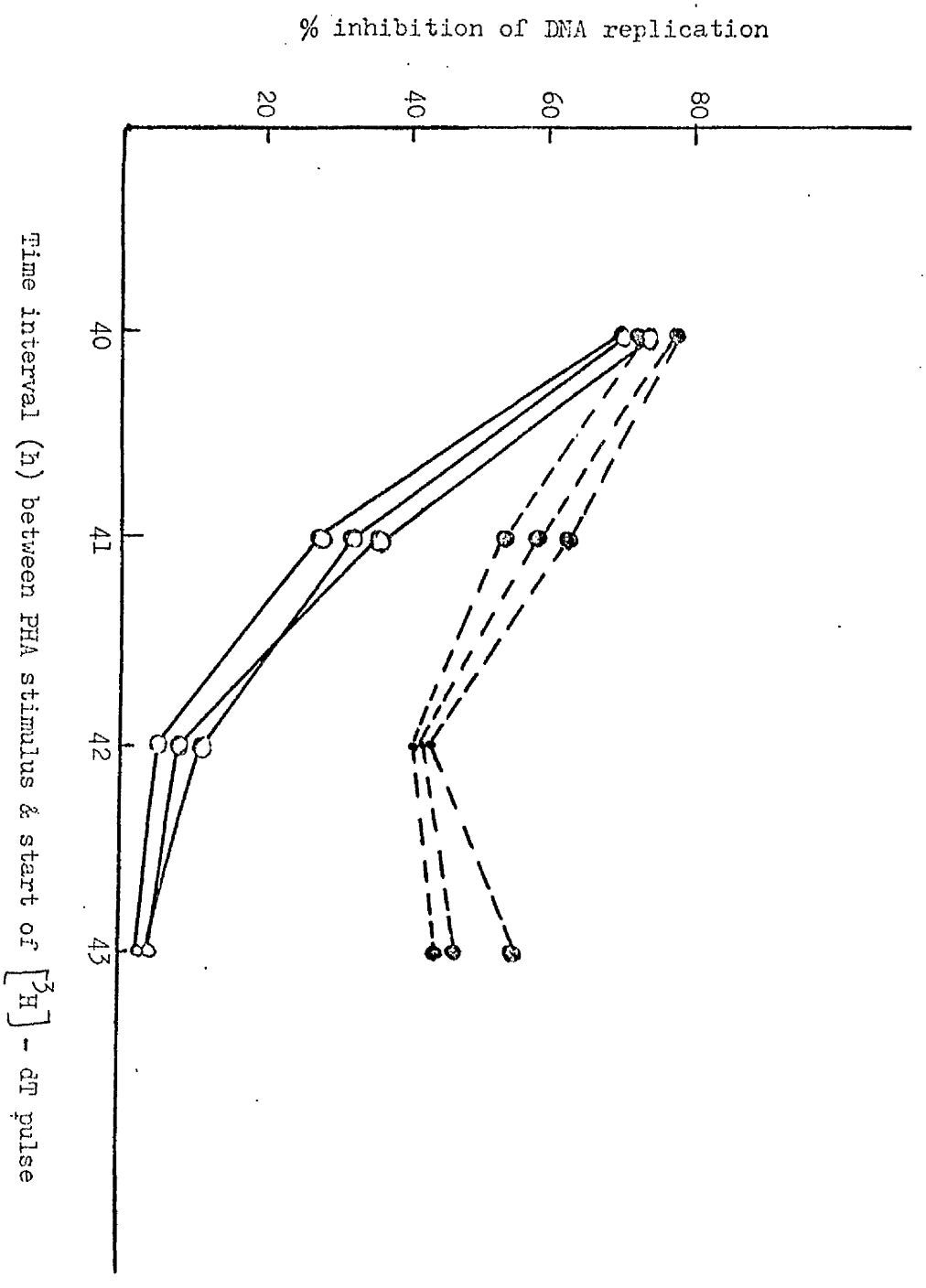
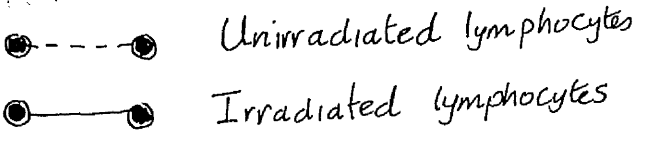

% inhibition of DNA replication
20
40
60
80
Time interval (h) between PHA stimulus & start of [3H] - dT pulse
40
41
42
43

Fig. 25 Time course of $[^3H]$ - dT incorporation into the acid-insoluble fractions of both u.v. irradiated and unirradiated PHA treated lymphocytes derived from normal individuals. Setting up the cultures, the amount of PHA added and the irradiation procedure were the same as described in the legend to Fig. 24. Immediately after irradiation (100 $J.m^{-2}$) or mock irradiation, cultures were incubated at 37°C and received an hourly pulse of $[^3H]$ - dT (0.5 $\mu$Ci/ml, 21 Ci/mmole) at the times shown. After incubation with the label, cultures were washed, fixed and assayed for $[^3H]$ - dT incorporation as described in methods section 2.1.6. The counting efficiency was determined by the channels ratio method.



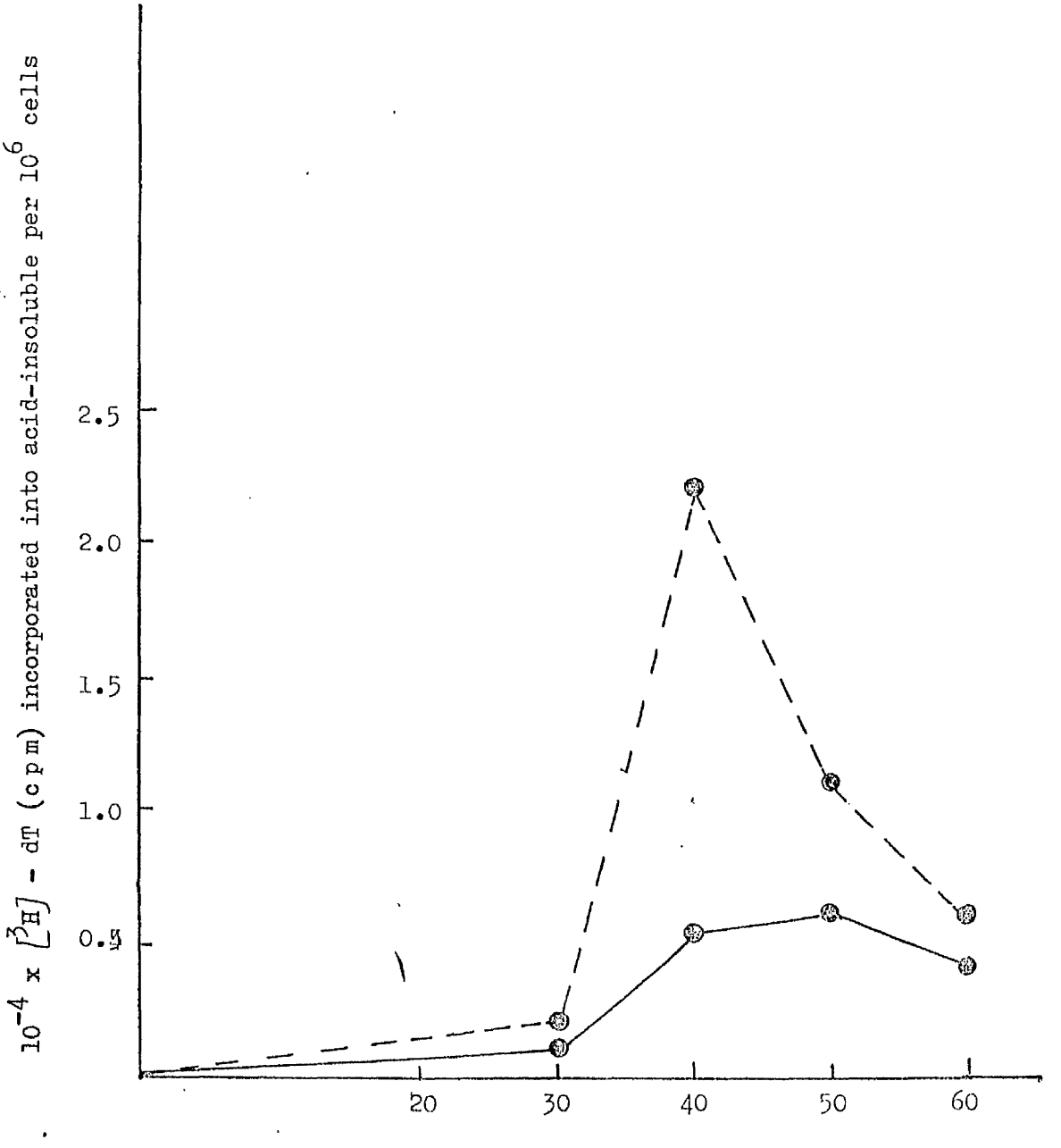
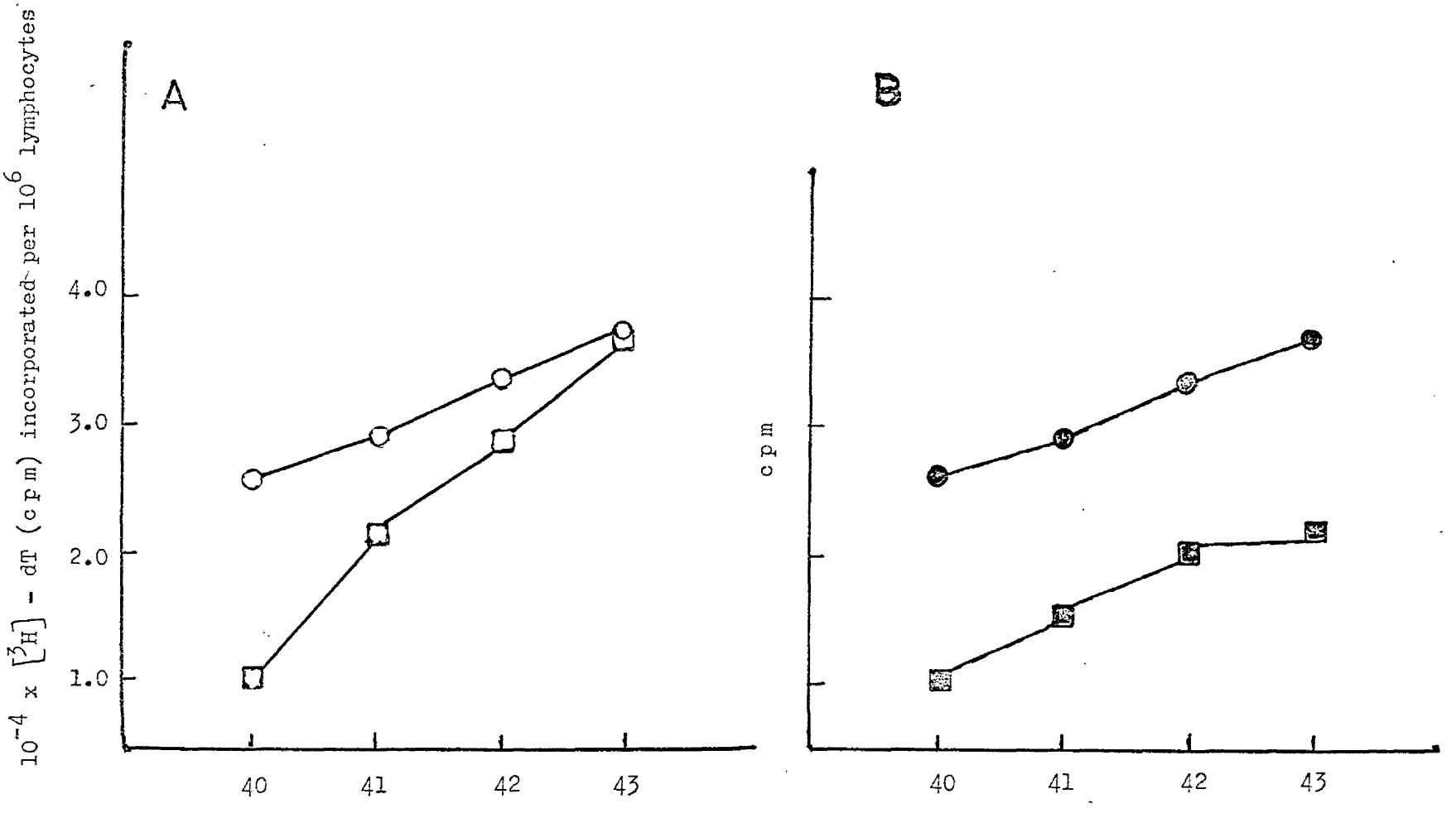

$10^{-4}$ x [$^3$H] - dT (c p m) incorporated into acid-insoluble per $10^6$ cells
2.5
2.0
1.5
1.0
0.5
20
30
40
50
60
Time interval (h) between PHA stimulus and start of [$^3$H] - dT pulse

Fig. 26 Time course of DNA repair, measured as the % recovery in DNA replication inhibited by u.v. light, in PHA stimulated lymphocytes derived from AK patient and age-matched normal individual. Setting up the cultures, PHA treatment of the cells and irradiation procedure were the same as described in the legend to Fig. 24. Immediately after irradiation (100 $J.m^{-2}$) or mock irradiation, cultures were incubated at 37°C and received an hourly pulse of [$^3$H] - dT (0.5 $\mu$Ci/ml, 21 Ci/mmole) at the times shown. After incubation with the label, cultures were washed, fixed and assayed for [$^3$H] - dT incorporation as described in methods section 2.1.6. The datum is expressed as the average [$^3$H] - dT (c p m) incorporated into duplicate irradiated cultures compared to that incorporated into unirradiated controls from the same donor. The counting efficiency was determined by the channels ratio method.

| | |
|---|---|
| A | Normal lymphocytes |
| B | AK lymphocytes |
| ○ | Unirradiated normal lymphocytes |
| ● | Unirradiated AK lymphocytes |
| □ | Irradiated normal lymphocytes |
| ■ | Irradiated AK lymphocytes |


A
B
$10^{-4}$ x [$^3$H] - dT (cpm) incorporated per $10^6$ lymphocytes
4.0
3.0
2.0
1.0
cpm
40
41
42
43
40
41
42
43
Time interval in (h) between PHA stimulus and start of [$^3$H] - dT pulse

It appears that cells from L.F. patients have a similar rate of DNA repair to that in AK cells. This has only been shown using the autoradiographic method (see Table 12), but the results agree with the report by Beighlie and Teplitz (1975) that L.F. cells show 50% repair deficiency compared to normal controls.

The cells from B.C.C. and M patients, (the genetics of which have not yet been studied) show a rate of DNA repair, very similar to that of age-matched normal individuals (Table 12 & 13).

Table 12 U.v. induced DNA repair in lymphocytes derived from lupus erythematosus, basal cell carcinoma and age-matched normal individuals, measured autoradiographically. The experimental details and analysis of the results are the same as described in the legend to table 6. The % of cells in S-phase was less than 1 in all cultures.

| Blood type | Mean grain count per cell irradiated at | |
|---|---|---|
| | 5.0 $J.m^{-2}$ | 20 $J.m^{-2}$ |
| LE | 6.9 ± 0.32 | 10.4 ± 0.49 |
| NL | 15.8 ± 0.52 | 24.3 ± 0.91 |
| LE | 8.5 ± 0.44 | 12.1 ± 0.59 |
| NL | 16.6 ± 0.62 | 26.4 ± 0.95 |
| BCC | 17.4 ± 0.57 | 23.8 ± 0.91 |
| NL | 18.8 ± 0.64 | 25.4 ± 0.93 |

Table 13 U.v. induced DNA repair in lymphocytes derived from patients with basal cell carcinoma, melanoma and age-matched normal individuals, measured by the total incorporation method. The experimental details and working out the results are the same as described in the legend to table 11.

N D = Not determined

| Blood type | Total $[^3H]$ - dT (dpm) incorporated into $10^6$ unirridiated cells in the presence of OH-urea | | DNA repair measured as the difference in $[^3H]$ - dT (dpm) incorporated per $10^6$ cells between irridiated (20 $J.m^{-2}$) and unirradiated cells from the same donor | | % DNA replication inhibited by OH-urea |
|---|---|---|---|---|---|
| | Duplicate 1 | Duplicate 2 | Duplicate 1 | Duplicate 2 | |
| B.C.C. | 1392 | 1179 | 5639 | 5124 | 74.3 |
| N L | 1412 | 1359 | 5271 | 5432 | 78.2 |
| B.C.C. | 1474 | 1462 | 4966 | 5277 | 80.9 |
| N L | 1506 | 1428 | 4867 | 4803 | 79.6 |
| B.C.C. | 1370 | | 5320 | 5429 | N.D |
| N L | 1281 | | 5338 | 5106 | N.D |
| M | 1564 | 1432 | 5295 | 5531 | 81.0 |
| N L | 1275 | 1317 | 4988 | 5318 | 76.8 |
| M | 1319 | 1207 | 4806 | 5065 | 75.1 |
| N L | 1345 | | 5032 | 5216 | N.D |

## 4. DISCUSSION

## 4.1 Methods for studying DNA repair

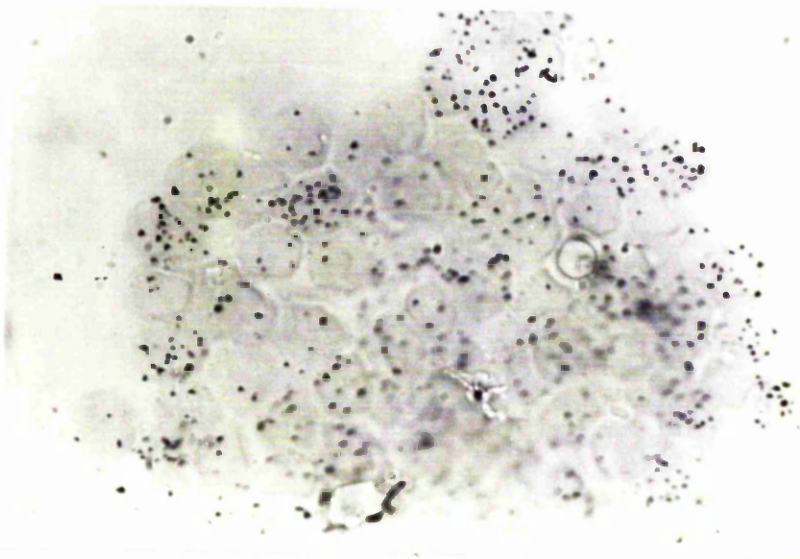
At the start of this project, the only established methods for the study of u.v. induced DNA repair were autoradiography (see methods section 2.1.5) and procedures based on the physical separation of repaired DNA from replicated DNA on a caesium chloride equilibrium density gradient (see results section 3.1.8). The work in mammalian cells, has been mostly carried out in growing cell populations in culture such as fibroblasts derived from skin biopsies. However, it is difficult to quantitate the extent of u.v. induced DNA repair in dividing cells due to inadequate resolution of DNA replication and DNA repair as described below. This problem can be overcome by using non-dividing cells such as peripheral blood lymphocytes. The practical advantages of lymphocytes over fibroblasts as a system for the study of DNA repair in patients with different clinical conditions were indicated earlier (see introduction section 1.6).

The method using caesium chloride gradients is expensive and time consuming and is therefore unsuitable for the routine assay of u.v. induced DNA repair in investigations of sun-sensitive conditions or in clinical diagnosis. Also, it is difficult to make the estimation quantitatively accurate since it depends on the quantitative isolation of DNA in each determination and on the complete resolution of the repaired and replicated DNA in the CsCl gradient. Such resolution is often inadequate because of the relatively large amount of replication (even after u.v. irradiation) and the small amount of repair synthesis.

The examination of DNA repair by autoradiography on the other hand, offers the advantage of looking at the populations of cells visually. This makes it possible to determine variation in repair activity among the population (see Figs 11, 12 & 13). However, the distinction between u.v. induced DNA repair in the non S-phase cells and DNA replication in these cells whose S-phase replication is partially inhibited by u.v. irradiation (Bootsma and Humphrey 1968) may be difficult at very high u.v. doses ( $>20$ J.m$^{-2}$) as reported by Cleaver (1967) and Epstein et al (1968). This problem is again eliminated when non-dividing cell populations are used for the study of DNA repair. There are other disadvantages associated with the autoradiographic method, the first of which applies specifically to its use with lymphocytes.

1) There is difficulty in obtaining a fixed cell population free of cell clumping (see Plate 7). This problem which has been encountered by other workers (Frey-Wettstein et al 1969), is caused by the fixation procedure and inevitably leads to overlapping of cells on the slides. In such preparations, grains are counted over nuclei of cells lying apart as there is no evidence to suggest that the aggregated cells are different (in relation to their DNA repair activity) from the single cells. Indeed the grains counted over those cells in clumps which can be counted are indistinguishable from those over the single cells. However, for photographic record (Plate 5), fields of dispersed cells are not very suitable because they contain too few examples.

Plate 7 Autoradiograph of a clump of normal lymphocytes exposed to 5.0 $J.m^{-2}$ and incubated with [$^3$H] - dT for 4 h at 37°C. For experimental details see the legend to Fig. 9



2) The method is more time consuming (10 days plus the time required to count the grains over sufficient cells compared with 24 h required to complete the total incorporation method.

In this work attempts were made to devise an easy, fast, reliable and inexpensive method for the estimation of u.v. induced D.N.A repair in human lymphocytes. One method, which fills these criteria adequately, is the total incorporation method (see methods section 2.1.6). It is an expensive modification of a technique first described by Robbins et al (1970).

Most of the cells in a lymphocyte culture (prepared as described in methods section 2.1.1) do not divide. However there is a very small percentage of cells which are in S-phase and incorporate $[^3H]$ - dT. This incorporation into replicated DNA masks the incorporation of $[^3H]$ - dT into the other 99% non-dividing, u.v. stimulated lymphocytes, undergoing repair synthesis. For this reason, OH-urea is used in the total incorporation method to inhibit DNA replication.

OH-urea inhibits semi-conservative DNA synthesis in a variety of cell types (Sinclair 1965; Doiffer and Tolmach 1967; Cleaver 1969a), but not u.v. induced $[^3H]$ - dT incorporation into Hela cells (Cleaver 1969a) or human lymphocytes (Evans and Norman 1968) during short exposures (3 h or less) of the cells to the drug. However, it has been reported (Ben-Hur and Ben-Ishai 1971) that extended exposure of Hela cells to OH-urea interferes with the repair of their u.v. damaged DNA. This interference is most probably due to the action of OH-urea on the enzyme ribonucleoside diphosphate reductase, which reduces ribonucleotides to

deoxyribonucleotides (Neuhardt 1967; Moore 1969). Either the pre-existing pools, or the residual reduced rate of deoxyribonucleotide synthesis, or both must be adequate to support normal levels of DNA repair synthesis for 3 h, but inadequate to allow continued repair or replication (where the rate of DNA synthesis is much higher, approximately 100 fold, Cleaver 1970). However, results presented by Robbins and Kraemer (1972a) in a much more extensive investigation showed that OH-urea has little or no effect on DNA repair synthesis in lymphocytes derived from normal individuals and XP patients even after 3 h. Even when the final concentration of OH-urea was increased from $1.25 \times 10^{-3}$M to $5.0 \times 10^{-3}$M, there was no decrease in the amount of prolonged (up to 23 h) u.v. induced $[^3H]$ - dT incorporation into XP lymphocytes. Furthermore, it was shown by the same authors that when OH-urea was added simultaneously with the $[^3H]$ - dT 3 h before terminating the experiment, rather than it being continuously present from the time of irradiation, incorporation of $[^3H]$ - dT into normal lymphocytes still ceased completely by the end of the 6th hour after irradiation, while incorporation into XP continued to the end of the 21st hour. Robbins and Kraemer (1972a) also estimated the u.v. induced DNA repair autoradiographically in the absence of OH-urea in lymphocytes derived from XP patients and normal subjects. Under these experimental conditions, repair was complete in the lymphocytes from normal patients in the same time (6 h), while that in lymphocytes derived from XP patients took much longer (21 h). It is not clear why the data of Robbins and Kraemer (1972a) disagree with those of Ben-Hur and Ben-Ishai (1971), but results presented in this work have confirmed

that time course of DNA repair is unaffected by OH-urea in normal and AK lymphocytes. Also the difference in repair activity between normal and AK cells is similar when measured by autoradiography in the absence of OH-urea and by the total incorporation method in the presence of the drug.

The reason for the selective action of OH-urea in inhibiting DNA replication, but not DNA repair, is not yet fully understood. However, it seems reasonable to accept as a working hypothesis that OH-urea reduces the rate of deoxyribonucleotide synthesis to such a level that it is unable to support the fast rate of DNA replication, but can sustain the much slower and smaller amount of synthesis required for DNA repair. The amount of thymidine incorporated into the DNA of $10^6$ lymphocytes as a result of u.v. induced DNA repair synthesis, estimated from Fig. 21, is 0.51 p mole/h in normal lymphocytes and 0.27 p mole/h in AK lymphocytes. The cellular dTTP pool (which was shown to be the same in both cell types, see Fig. 21) is 0.65 p mole/$10^6$ cells. Such a dTTP pool size chosen without slow replacement is therefore adequate to maintain DNA repair synthesis for 1.25 h and 2.4 h in normal and AK lymphocytes respectively.

The value of 0.65 p mole/$10^6$ cells for the dTTP pool size in both AK and normal lymphocytes is in fairly good agreement with that found in other cell types. Adams et al (1971) reported that the dTTP pool size in stationary mouse L cells is 3 p mole/$10^6$ cells. This value is reasonably close to that obtained in lymphocytes, when corrected, for the size difference between the two cell types (i.e. L cells are 3.4 times larger than lymphocytes), and also the

fact that there is an even smaller percentage (<1%) of S-phase cells in lymphocyte populations than that present in stationary L cells.

The advantages of the total incorporation method are:-

1) It is relatively fast (i.e. the results are available 24 h after collecting the blood sample.
2) The reproducibility of the method is very good (see tables 9, 10 and 11).
3) Several assays can be performed simultaneously.
4) The final results can not be influenced by subjective bias (which is always a difficulty with autoradiographic grain counting). The method has been applied in this work to the examination of DNA repair in a large number of AK patients and age-matched normal subjects. It has also been applied to the preliminary investigation of u.v. induced DNA repair in lupus erythematosus, melanoma and basal cell carcinoma. The method is quite suitable either for routine diagnostic use or for familial surveys aimed at establishing the genetic bases of AK.

Another method which can be used to estimate u.v. induced DNA repair is a novel approach based on the following principles:-

1) Lymphocytes which are generally at rest in $G_1$ can be induced into a division cycle with the mitogen PHA (Nowell 1960; McIntyre & Ebaugh 1962).
2) U.v. irradiation of dividing cells, inhibits their S-phase DNA replication (Cleaver 1967; Painter et al 1970 and see results section 3.11).

3) After the damage caused by u.v. irradiation is repaired, DNA replication resumes (Rasmussen et al 1970).

The stimulation of lymphocytes with PHA leads 30 h later to the incorporation of [$^3$H] - dT into DNA. This incorporation is maximal 48 h after the addition of PHA (see Fig. 23).

The inhibition of S-phase DNA synthesis with u.v. light is thought to be due to the introduction of thymine dimers or other photoproducts (see introduction section 1.3.3), which block the progress of the DNA polymerase (Painter 1974). However, after the removal of the dimers probably by the joint action of excision repair and post-replication repair mechanisms (see introduction sections 1.4.3 & 1.4.4), DNA synthesis resumes and this resumption can be taken as an indirect measure of successful DNA repair.

One difficulty with this method is that stimulation with PHA is particularly sensitive to slight changes in the pH of the medium or in the temperature of incubation. Such variations can cause a delay in the time course of stimulation but with care, they can be avoided. The period of 40 h incubation of lymphocytes with PHA prior to u.v. irradiation, was found to provide the best conditions for the study of DNA repair. This is based on two criteria:-

1) The difference in [$^3$H] - dT incorporation into irradiated and unirradiated, PHA treated human lymphocytes, is greatest after 40 h incubation with the mitogen, hence greater percentage inhibition of DNA replication by u.v. light can be achieved (see Fig. 25).

2) The rate of repair (incorporation of $[^3H]$ - dT per unit time) is fairly constant over a 4 h period for both normal and AK cells (see Fig. 26) which is in agreement with earlier results (see Fig. 8).

Under these conditions, u.v. induced DNA repair has been studied in lymphocytes derived from AK patients and age-matched normal subjects and the results obtained by this method confirm the earlier finding that the rate of u.v. induced DNA repair in AK lymphocytes is about 50% that in normal lymphocytes (see Fig. 24). Like that estimated by the total incorporation method, the repair in normal lymphocytes measured by this method is complete after 4 h, following u.v. irradiation (see Figs 26A & 8).

However, prolonged incubation (beyond 4 h) of PHA treated lymphocytes, following u.v. irradiation, makes the analysis of u.v. induced DNA repair more difficult to interpret. This is most probably due to the treatment cells received during the irradiation procedure (see methods section 2.1.7). Thus, lymphocytes incubated uninterruptedly with PHA, show a maximum incorporation of $[^3H]$ - dT at 48 h (see Fig. 23A), while similarly treated lymphocytes except for the interruption during mock-irradiation, show a much reduced level of $[^3H]$ - dT incorporation at approximately the same time (50 h) see Fig. 25. It is also evident from Fig. 25 that u.v. irradiation of PHA treated lymphocytes gives rise to a smaller percentage of inhibition in DNA replication after 40 h. This may be due to the greater resistance of a population of cells which take longer to enter S-phase after PHA treatment. Such a population of cells

may have different survival characteristics as well as a different response to PHA (Evans and Norman 1968). Alternatively, the DNA replicating late in the S-phase may be G C rich and hence less sensitive to thymine dimer formation after u.v. irradiation, or the increased amount of DNA may shield the remaining unreplicated sections from the damaging effects of u.v. light.

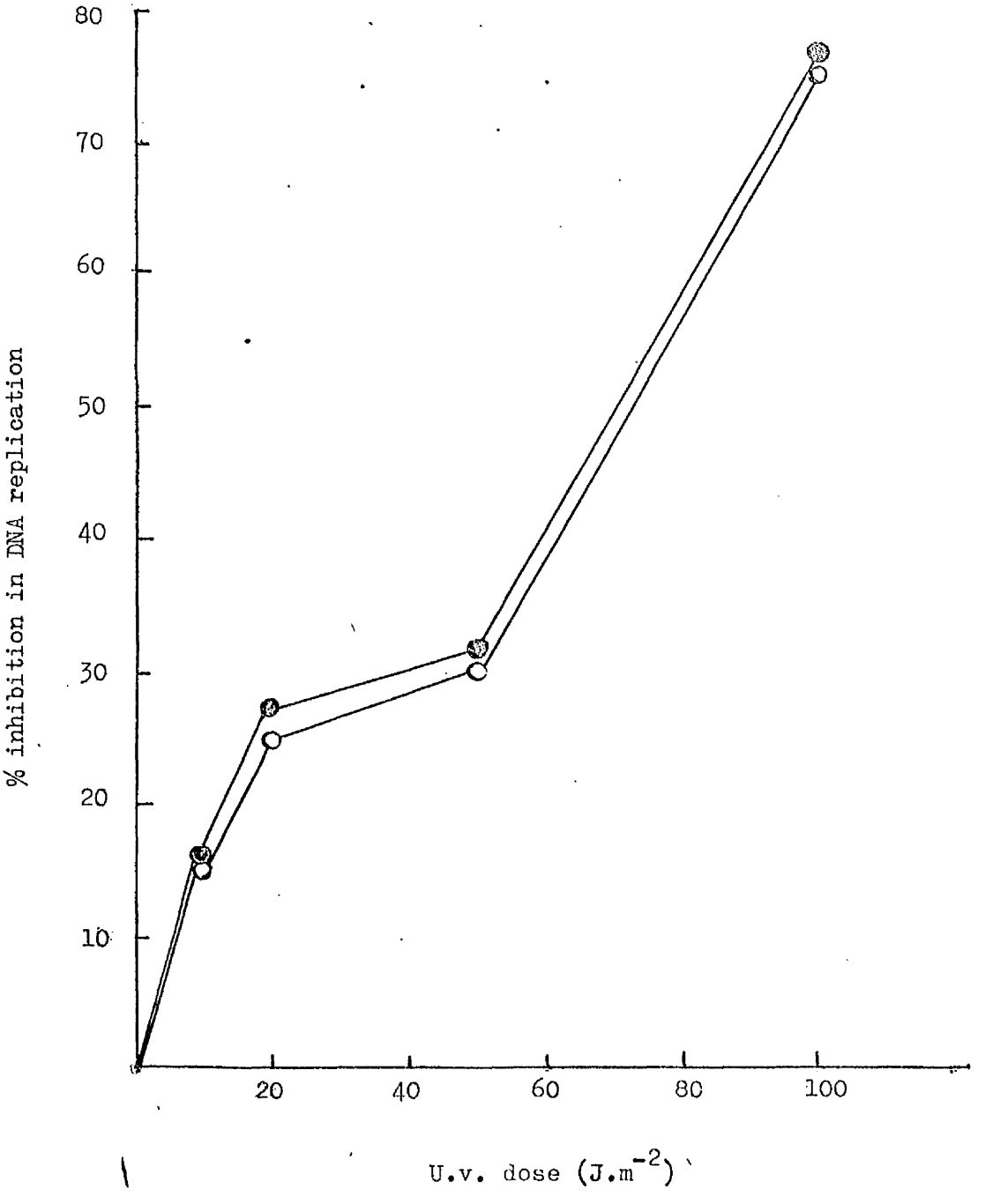
However, the results obtained by this indirect method, showing that the initial rate of u.v. induced DNA repair in AK lymphocytes averages 50% that of normal lymphocytes, compare well with those obtained by autoradiography (48.7% of normal) and by the total incorporation method (45.5% of normal). This suggests that the inhibition of DNA replication is directly proportional to the number of thymine dimers formed by u.v. irradiation, assuming that thymine dimers are the major photoproduct of u.v. irradiated mammalian cells (see introduction section 1.3.3).

The u.v. dose response curves for inhibition of DNA replication in PHA stimulated lymphocytes derived from AK patients and age-matched normal individuals are very similar and appear to be biphasic (Fig. 27). The first part of the curve is similar to the earlier dose response curves of repair synthesis obtained by both the autoradiographic and the total incorporation methods (see Figs 9 & 10). The similarity of the curves at u.v. doses up to 50 $J.m^{-2}$ further suggests that inhibition of DNA replication is caused by the same types of photoproducts which give rise to repair synthesis. The other part of the curve, at higher u.v. doses, may be due to the formation of other photoproducts such as interstrand crosslinks (which are usually formed at high u.v. doses, Marmur and

Fig. 27 U.v. dose response curves of DNA replication in PHA treated lymphocytes derived from AK patients and age-matched normal individuals. Setting up the cultures, the amount of PHA added and the irradiation procedure were the same as described in the legend to Fig. 24. Cultures were irradiated at different doses ranging from 0 to 100 $J.m^{-2}$ or mock irradiated and incubated immediately with $[^3H]$ - dT (0.5 $\mu$Ci/ml, 21 Ci/mmole) at 37°C for 1 h. After incubation with the label, cultures were washed, fixed and assayed for $[^3H]$ - dT incorporation as described in methods section 2.1.6. The datum is expressed as the percent reduction in the average $[^3H]$ - dT (c p m) incorporated into duplicate irradiated cultures as compared to that of duplicate unirradiated controls from the same donor. The counting efficiency was determined by the channels ratio method.

○ Normal patient lymphocytes

● AK patient lymphocytes


80
70
60
50
40
30
20
10
% inhibition in DNA replication
20
40
60
80
100
U.v. dose (J.m$^{-2}$)

Grossman 1961). The repair of such damage may require a mechanism, similar to that occurring in bacteria (Cole 1973), which involves sequential excision and strand exchanges between homologous duplexes (see introduction section 1.4.4).

4.2 $[^3H]$ - dT incorporation as a measure of DNA synthesis

An important problem associated with comparative studies of u.v. induced DNA repair, based on $[^3H]$ - dT incorporation which has been ignored by previous authors, is the possible effects which could be caused by different dTTP pool sizes in different cell types. Different pool sizes of endogenously synthesised dTTP would, for example, cause different dilutions of the specific activity of $[^3H]$ - dTTP derived from the exogenous $[^3H]$ - dT. This in turn would give rise to a different extent of $[^3H]$ - dT incorporation into DNA of the different cells, even though the rates of DNA synthesis were the same. In this study circumstantial evidence from early experiments suggested that the dTTP pool sizes are similar in AK and normal lymphocytes. The rates of incorporation of $[^3H]$ - dT into PHA stimulated lymphocytes from AK patients and normal individuals are very similar (see Fig. 23) and the inhibition of incorporation by u.v. light is the same in both cell types, while the recovery rates (i.e. repair) are different (see Fig. 24). However, the dTTP pool sizes were determined for both cell types and it was shown (Fig. 21) that, while the true rates of DNA synthesis in the two cell types are different, the dTTP pool sizes are the same.

## 4.3 AK cells have reduced DNA repair activity

Under the conditions used to compare repair activity by the total incorporation method, the initial rate of u.v. induced DNA repair in lymphocytes derived from AK patients appears, from the work described in this report, to be about 50% that of lymphocytes derived from age-matched normal subjects. Eventually, however, the AK cells complete a quantitatively similar amount of DNA repair synthesis to that found in normal cells. These conclusions are based on several different experimental approaches.

The finding that the extent of DNA repair, given sufficient time, is similar in AK cells to that in normal cells (Fig. 22), is analogous to that of Robbins and Kraemer (1972a), who showed that XP lymphocytes with reduced rate of DNA repair (25% of normal) measured over the first 3 h, following u.v. irradiation, eventually complete normal levels of repair in 21 h. The more greatly reduced rate of repair and the consequent longer time required to complete the repair in XP cells compared to AK cells is consistent with the increased clinical severity of the XP conditions.

With the exception of one value (where the rate of repair in AK cells was found to be 63% that of the normal), the results from all three methods used to examine DNA repair are strikingly similar. The rates of repair in cells from AK patients are all in the range 40 - 50% that in cells from normal patients. This not only means that the levels of repair in the cells of different AK patients was consistent but also that the repair in the cells of the age-matched control subjects was equally consistent. It is perhaps surprising

that all clinically diagnosed AK proved to be AK on the basis of the repair assays and that no repair deficient, pre-AK patients were discovered. This must be partly due to the low frequency of AK among the population (< 1%) so that the probability of selecting a pre-AK subject in less than 50 normal subjects is small. Furthermore all the normal subjects were age-matched and therefore had had adequate opportunity to develop AK symptoms if they were so predisposed. The absence of normal patients with low repair or AK patients with normal repair in this study confirms the accuracy of the clinical diagnosis. However, further work is needed to establish the variation of reduced repair in AK patients lymphocytes, to determine if the pre-AK condition can be identified by the repair assay and also to show that reduced levels of repair occur in cells from AK patients skin, the primary site of their disease.

4.4 Is reduced DNA repair activity the common basis of XP and AK?

The first indication of the biochemical defect of XP was the demonstration that XP cells were abnormally sensitive to inhibition of growth by u.v. light (Gartler 1964). This was followed by the discovery that XP cells resemble *uvr* bacteria in their reduced levels of excision repair of u.v. damage because of defects at an early step of repair (Cleaver 1968, 1969b). Reduced levels of repair were found in both the common forms of XP and the rarer de Sanctis-Cacchione syndrome (Setlow *et al* 1969; Bootsma *et al* 1970). The early observation of u.v. sensitivity by Gartler (1964) was later confirmed by examining u.v. survival curves, which showed that the

genetic defect in XP enhanced the lethality of a given u.v. dose by a factor of 4 - 10 (Goldstein 1971).

The repair deficiency in u.v. irradiated XP cells has been associated with the extreme sun-sensitivity and increased frequency of skin cancer, and regarded as a possible factor in the etiology of the disease (Cleaver 1968). There is a considerable variation in the reduced repair activity of cells from different XP patients (see introduction section 1.5) which correlates with the clinical severity of the disease (Bootsma et al 1970).

The repair deficiency in cells derived from AK patients strongly suggests that AK, like XP is due to a genetic defect. While the genetic basis of XP is known, as yet no familial studies of the inheritance of AK has been undertaken. The total incorporation method (see methods section 2.1.6) could be successfully deployed for the study of the genetic basis of AK, by estimating u.v. induced DNA repair in people diagnosed as AK patients and their relatives. Such a study should show whether or not the repair deficiency is necessarily due to a genetic defect.

However, it seems likely that reduced DNA repair in AK cells is caused by a genetic factor similar to that in XP cells. In bacteria (see introduction section 1.4.3), the presence of a mutation in any one of the three unlinked loci, uvr A, uvr B, uvr C, reduces the ability of cells to excise thymine dimers from their u.v. irradiated DNA. Mutations in more than one of the loci reduces the level of DNA repair still

further (Howard-Flanders 1975). By analogy, it is possible that the very much lower levels of u.v. induced DNA repair in XP (Bootsma et al 1970), as compared to AK is due to a similar effect as that found in bacteria (i.e. it is possible that AK is the result of a mutation in one locus (equivalent to uvr A, B or C) and that two AK homozygotes (as phenotypically normal heterozygotes) may give rise to an XP progeny with mutations in two loci).

The results presented in this report suggest that the repair deficiency in AK lymphocytes is due to a defective excision repair mechanism. This conclusion is based on the fact that, when DNA repair was studied in non-dividing lymphocytes, the u.v. irradiated cells were only briefly (few minutes) exposed to low levels of photoreactivating light (i.e. laboratory illumination) and for the rest of the 4 h period incubated in the dark, where photoreactivation cannot operate (see introduction section 1.4.2). Fig 8 shows the time course of u.v. induced DNA repair in lymphocytes derived from AK patients and age-matched normal individuals, where it can be seen that the repair proceeds at an approximately constant rate during the 4 h incubation period and it is these rates which are different in AK and normal cells.

However, this evidence does not rule out the participation of a post-replication mechanism in the repair of u.v. induced damage, but it is difficult to envisage a role for such a mechanism in non-dividing cells (see introduction section 1.4.4).

It is possible that DNA of non-dividing cells contains repetitive segments which could allow a low level of repair activity analogous to post-replication repair. However, the results of u.v. induced DNA repair, obtained in PHA stimulated lymphocytes of AK and normal patients, where post-replication repair is most likely to occur, show the same reduced rate of DNA repair in AK cells. It would seem reasonable therefore to assume that both methods are measuring the same repair process i.e. excision repair.

The most likely cause of the decreased u.v. induced DNA repair in cells derived from AK patients is a deficiency or impairment of activity of one of the enzymes involved in excision repair in these cells, because the exonuclease, the DNA polymerase and the ligase may be involved in other processes (e.g. recombination, replication). It is further likely that the deficiency lies in an endonuclease specific for u.v. damaged DNA (c.f. the molecular basis of XP).

## 4.5 DNA repair in conditions other than AK

Reduced levels of u.v. induced DNA repair have been reported for cells from patients with sun-sensitive conditions such as, photodermatoses (Norkay et al 1973), Franconi's anaemia (Poon et al 1974), lupus erythematosus ( Beighlie and Teplitz 1975) and Down's syndrome (Lambert et al 1976b). These conditions are also associated with an increased incidence of malignancy, which further substantiates a relationship between defective DNA repair and carcinogenesis.

In this work, preliminary investigations also showed that lymphocytes derived from patients with lupus erythematosus have reduced levels of u.v. induced DNA repair, as compared with lymphocytes derived from normal patients (see Table 12). Deficiency in repair implicated with genetic factors, has been reported for cells derived from patients with lupus erythematosus (Beighlie and Teplitz 1975). The results presented in Table 12 which agree well with those reported by Beighlie and Teplitz (1975) lend further support for the hypothesis that deficiency in repair is caused by genetic defects. The estimation of u.v. induced DNA repair in melanoma and basal cell carcinoma showed that cells from patients with these conditions have very similar levels of repair to that in normal cells (see Table 12 & 13). This shows, not unexpectedly, that tumours can arise from causes other than DNA repair deficiency.

Not all forms of neoplasm stem from deficiency in DNA repair, for example, u.v. irradiated Hela cells have been shown to have a normal level of DNA repair (Cleaver and Painter 1968).

Further work on cells from patients with sun-sensitive conditions may lead to a better understanding of the role of DNA repair in skin carcinogenesis.

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