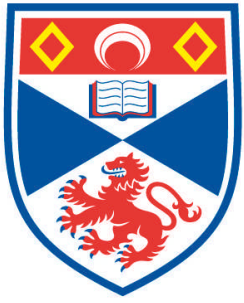
EFFECTS OF TRYPSIN ON CELLULAR, CHROMOSOMAL AND DNA DAMAGE INDUCED BY X-RAYS

Elizabeth A. Sprunt

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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EFFECTS OF TRYPSIN ON CELLULAR, CHROMOSOMAL AND DNA DAMAGE INDUCED BY X-RAYS

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Thesis submitted for the degree of

PhD in Radiation Biophysics

to the Department of Biology and Preclinical Medicine

University of St Andrews

October 1989

Declaration

I, Elizabeth A. Sprunt, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any degree or professional qualification.

Signed E. Sprunt Date 24/10/89

I was admitted to the faculty of Science of the University of St.Andrews under Ordinance general No. 12 on 10/86 and as a candidate for the degree of Ph.D. on 10/87

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ABBREVIATIONS

ds(b) - double strand (break) of DNA

EDTA - ethylene diamine tetra-acetic acid

EB - ethidium bromide

LD - lethal damage

NLS - sodium lauryl sulphate

PLD - potentially lethal damage

SLD - sub lethal damage

SDS - sodium dodecyl sulphate

ss(b) - single strand (break) of DNA

trypsin - (generally) the trypsin solution used routinely to produce cell suspensions, including the EDTA/buffer salts solution in which the trypsin was dissolved

buffer - (generally) the EDTA/buffer salts solution in which trypsin is dissolved for routine passaging

DW agarose - low gelling temperature agarose made up in distilled water

PBS agarose - low gelling temperature agarose made up in PBS

ABSTRACT

When cells are trypsinized before irradiation, potentiation of cell killing is seen; this is known as the 'trypsin effect'. The trypsin effect is re-examined here in the light of experiments in which enzymatic modifications of DNA in permeabilized cells has become a powerful experimental tool (Bryant *et al*, 1978, Ahnstrom and Bryant,1982; Natarajan *et al*, 1980; Bryant, 1984, 1985; Natarajan and Obe, 1984) and where in some cases it is suspected that trypsinization as part of the technique could significantly alter cell membrane permeability and chromatin structure (Obe *et al*, 1985; Obe and Winkel, 1985; Bryant and Christie, 1989).

The trypsin effect was investigated at various cellular levels, assaying for cell survival (to verify the potentiation), anaphase chromosomal aberrations, DNA damage and repair and lastly using a nucleoid assay to investigate the effect of trypsin on DNA-nuclear matrix interactions. Each of these are considered in separate chapters as individual studies, then all compared in the final discussion.

A small potentiation effect of X-ray damage on cell killing was seen when using Chinese Hamster Ovary (CHO) cells but no potentiating effect was foung in the murine Ehrlich ascites tumour (EAT) cell line. Trypsinization was found to increase the number of X-ray induced chromosomal anaphase abnormalities in EAT cells.

To investigate the possibility that the basis of the trypsin effect lies in its action at the DNA level, further experiments were performed to monitor DNA damage and repair using the DNA unwinding and neutral elution techniques. No difference was seen in the unwinding kinetics or in the DNA unwinding dose-effect curves for induction of DNA single strand breakage (ssb); when using neutral elution however,

treatment of cells with trypsin or buffer alone increased the incidence of X-ray induced double strand breaks (dsb) at higher doses.

Trypsinized EAT cells were found to repair ssb after 12 Gy less rapidly than those treated with buffer (EDTA) indicating an inhibitory effect of trypsin on repair. A progressive decrease in repair capacity with increase in time of trypsin treatment was seen.

The dsb repair kinetics as measured by the DNA unwinding technique after 50 Gy showed that either trypsin of buffer (EDTA) alone reduced the dsb repair rate, no difference between their repair kinetics being evident (this was also seen with neutral elution repair after 40 Gy). This indicates that the EDTA/buffer solution in which the trypsin is dissolved may also be contributing to the trypsin effect.

A new nucleoid assay was developed and used to investigate the effect of prolonged trypsinization and electroporation on nucleoid morphology.

The results confirm that routine trypsinization of cells enhances X-ray induced cell killing in some cell lines. It is postulated that this may occur by reducing the repair capacity of the cells rather than by increasing the amount of damage initially caused.

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Chapter 1

This first chapter provides a general introduction to the investigation and is applicable to all chapters; more specific detail is given in relevant chapters where necessary.

Reasons for using a trypsin/EDTA solution for routine cell passaging rather than an alternative method are first discussed. This solution is referred to as 'trypsin' in the text unless a distinction between the trypsin and EDTA is to be made.

The effect of trypsin on the cell is considered at progressively more detailed levels of cellular organisation from whole cell and membrane effects to the influence of trypsin on chromatin and DNA, followed by a discussion of what is known about the potentiation of X-ray damage to the cell by trypsin.

Finally the reasons for carrying out this study and the aims of the investigation are stated.

1. A comparison of cell suspension methods: why trypsin/EDTA is used

Cultured cells must be regularly diluted down into fresh growth medium (passaged) to keep the cells healthy and cycling, a unicellular suspension being made to allow counting and to discourage cell clumping. Those cells which grow in suspension such as Ehrlich ascites tumour (EAT) cells or which attach to the flask only loosely such as HeLa may be resuspended simply by agitating the flask then pipetting to separate any clumps. The fibroblastic cell lines however such as Chinese hamster ovary (CHO) and lung (V-79) grow firmly attached to the culture flask surface, cell suspension requiring enzymic digestion (trypsin, Pronase or collagenase), chelating agents (Versene, *ie* EDTA), or a combination of these. Less commonly, cells may be lifted from the flask using a manual method, *eg* scraping. All these techniques result in the release of low molecular weight components from the cells (Hebb and Chu, 1960; Levine, 1960; Litwin, 1973; Phillips and Terryberry, 1957) indicating damage to the cell but the amount of damage varies between methods; the various cell types also require different suspension methods to obtain optimum cell suspension with minimum damage.

There follows a discussion of the more common suspension methods, their advantages and disadvantages.

1.1 Scraping

Scraping cells from dishes using a rubber policeman, bacteriological loop or the sterile rubber end of a syringe plunger is extremely damaging to the cell and is not routinely used: plating efficiencies of 60-80% were found for trypsinized V-79 cells but this was found to be only 4% for cells scraped from the dishes (Sprunt, 1986). Another method is to mop the cells from the dish with a specially designed piece of sterile, perforated cellophane (Microbiological

Associated Inc.; Evans and Taylor, 1973). The effectiveness of this method is not known and it would not be viable for routine passaging.

1.2 Pronase

Pronase is the trade name of a crude preparation of proteolytic enzymes obtained from a broth culture of *Streptomyces griseus*, used in the manufacture of streptomycin (Gwatkin, 1973) and consists of 5 to 7 components, including one crude trypsinlike enzyme and 3 elastase enzymes. It disperses many cell lines (especially primary lines) more rapidly and efficiently than any other known enzymatic method and is more efficient than trypsin in digesting dead cells and cellular debris (Gwatkin and Thomson, 1964; Sullivan and Schafer, 1966; Weinstein, 1966; Foley and Aftonomos, 1970). No significant change in chromosome number, tetraploidy, breaks or gaps were noted in pronase treated cells (Weinstein, 1966). Its main disadvantage is that it is not inactivated by serum as is trypsin, and so the cells must be thoroughly washed before dispersing in growth medium. Even after washing, some residual enzyme remains at the cell surface (Foley and Aftonomos, 1970; Poste, 1971), which is probably the cause of contrasting reports of cell survival after pronase treatment (Kahn *et al*, 1965; Weinstein, 1966; Sullivan and Schafer, 1966; Foley and Aftonomos, 1973). Pronase is also inefficient at giving single cell suspensions of some cell lines such as HeLa, which tend to remain in sheets (Foley and Aftonomos, 1970; Kahn *et al*, 1965) and is therefore most commonly used to dissociate tissues prior to cultivation (Wilson and Lau, 1963; Gwatkin and Thomson, 1964) rather than to passage established cell lines.

1.3 Collagenase

The action of collagenase is very mild as it mainly acts on the collagen in the connective tissue surrounding cells rather than attacking the cell surface. Its main action is on collagen but even purified preparations contain contaminating enzymes (protease, esterase and polysaccharidase) as well as collagenase and will separate some cell types without large amounts of surrounding collagen (Hilfer, 1973). Its advantages are low cell damage, high percentage attachment and high plating efficiency (Hilfer, 1973) and so is used when cell viability is the priority, but does not give reliable single cell suspensions (*eg* Coon, 1966; Spooner, 1970) and so is usually used to break up lumps of tissue rather than in cell culture.

1.4 Trypsin

Trypsin was the first enzyme reported to disperse tissue samples (Rous and Jones, 1916) and is still one of the main cell dispersal methods for primary tissue disaggregation (Paul, 1975; Kruse and Patterson, 1973). Trypsin alone will not disperse all tissue types well due to its limited substrate specificity, and long incubation times can be necessary, resulting in cell damage; when using crystalline trypsin (the least aggressive) the cells tend to stick together in long strings, probably held together by DNA from damaged cells (Paul, 1975). Trypsinization is now, however, a standard part of the routine passaging procedure of continuous lines, especially when used in combination with other components such as EDTA (Puck and Marcus, 1955; Paul, 1975), and its action on cells has been widely investigated. Trypsinization does not initially result in a unicellular cell suspension (Gwatkin and Thomson, 1964); the clumps of cells must then be dispersed by pipetting. Rinaldini (1959) found no evidence of cell killing due to routine trypsinization, but after repeated trypsinizations within a short time the

proportion of viable cells decreased rapidly: 4 such trypsinizations left only 6% surviving cells. Trypsin is toxic at high concentrations (Rous and Jones, 1916) and affects various cell properties (Hodges *et al*, 1973; Puck and Marcus, 1955; Hebb and Chu, 1960) which are not thought to be important when routinely passaging. If cells are left in trypsin they will not flatten and attach to a surface, but will stay rounded and will not go back into cycle; those resuspended by scraping attach within 2 hours (Hebb and Chu, 1960; Feldman, 1955). The observed alterations of the cells due to trypsin revert to normal within 24 hours of trypsinization (Hebb and Chu, 1960). Cells are generally resuspended in fresh medium directly from the trypsin solution, as (unlike with Pronase) the traces of trypsin present do not adversely affect the cells and their attachment is better than if centrifuged out of medium (Litwin, 1973). The temperature at which trypsinization is carried out alters the effect of trypsin on the cell; trypsinization at 4°C produces less cell damage and a higher proportion of cells subsequently attach, but the cells must be left in trypsin for around 10 minutes longer than at 37°C and clumping is more extreme, making cell counting or clonogenic assay more difficult. Lengthy exposures to trypsin or excessive pipetting will increase the time taken for the cells to subsequently attach (Taylor, 1961). The pH of the trypsin solution must not be lower than pH 7.4 as during the course of trypsinization the pH drops so this will avoid the cells being in an acidic solution at the end (Litwin, 1973). The pH is also important for trypsin's activity. Below pH 7.0, trypsin is virtually inactive, while above pH 8.0, cells are progressively damaged by trypsin.

1.5 EDTA

EDTA is a chelating agent which, under the trade name Versene (0.02% EDTA in 0.85% saline) is successfully used on its own to form cell suspensions in some cell lines, but others such as human primary diploid

lines are greatly damaged by Versene treatment (Litwin, 1973). Lengthy Versene treatment or much post-treatment pipetting will induce a longer delay before cell attachment (Taylor, 1961).

1.6 Combinations of components

The more refined cell suspension techniques for primary and continuous cultures use a solution consisting of a mixture of components, several mild effects on different cell components producing a unicellular suspension in a shorter time with less cell damage than when using just one component *eg* a mixture of collagenase, trypsin and chicken serum (CTC) causes less cell damage than just collagenase for many cell lines, cell yield, attachment and cell growth all being improved (Coon, 1966). Trypsin has such a specific action that alone it can only cause limited proteolysis, so it is often used in conjunction with other components. Collagenase is often added in addition to trypsin when preparing primary cultures with a high collagenase content and where fibrous material present may trap cells and so reduce cell yield (Wiepjes and Prop, 1970; Montes de Oca, 1973). Trypsin is often used with EDTA for cultured cells as this generally increases the cell yield, reduces the dissociation time and gives a better unicellular suspension (Montes de Oca, 1973; Cox *et al*, 1970) although if the trypsin concentration is high, adding EDTA will reduce subsequent cell attachment (Taylor, 1961; Litwin, 1973). Trypsin and EGTA (ethylene glycol-bis (B-aminoethyl ether)N,N'-tetraacetic acid) has also been used for cell dispersal (Robb, 1973).

It is emphasised that not all cells respond similarly to these enzymes; fibroblastic cell lines such as the Chinese hamster ovary line are dispersed efficiently by a combination of trypsin and EDTA as was used in this investigation.

2. The properties and action of pure and commercial trypsin and EDTA

2.1 The properties and function of pure trypsin and EDTA

Trypsin is a fairly large enzyme (Mr 34,000) the most specific of the 7 proteases secreted by the pancreas into the small intestine. Only the peptide bonds at the carboxyl ends of arginine and lysine residues are hydrolyzed by trypsin, apart from simple ester derivatives which are also readily cleaved, particularly if they have an N^{α}-acyl substituent. Molecular 'fingerprinting' makes use of trypsin's specific action: molecules are digested with trypsin and the resulting fragments separated by two-dimensional chromatography or electrophoresis. An impression of the degree of relatedness of 2 enzymes can be gained from the observed similarity of their digests (Harriss and Perham, 1965).

The primary and tertiary structures of trypsin are known; 6 disulphide bridges maintaining its conformation (Bernhard, 1964; first sequenced by Walsh *et al*, 1964). Trypsin is known as a serine protease due to the uniquely reactive serine residue (Ser-183), while the adjacent aspartic residue (Asp-182) is important for specificity determination, both being found in the substrate binding site (Shaw, 1969).

The 7 pancreatic proteases have complementary specificities, so together they can efficiently fragment the proteins leaving the stomach where they have already been partially hydrolyzed. Trypsin has a very great activity, cleaving proteins when present at very low concentrations; native proteins are not broken down as efficiently as these partial digests.

An inactive precursor of trypsin, trypsinogen (the zymogen) is initially secreted from the pancreas which is later activated by the action of enterokinase, an enzyme secreted from the intestinal mucosa, or later autocatalytically. Also secreted in the pancreatic juice is an

inactive compound of trypsin known as trypsin inhibitor which prevents premature activation of trypsinogen, and may later be cleaved into active trypsin and another molecule by enterokinase or trypsin. Trypsin inhibitor forms again if these two molecules are brought together again.

EDTA is a chelating agent which binds to and therefore reduces the concentrations of the divalent cations Mg^{++} and Ca^{++} to vanishingly small amounts. If Ca^{++} are removed from the cell environment the cell attachment mechanism stops functioning as the necessary protein attachment is prohibited. EDTA is known as a hexadentate ligand as it may form as many as 6 dative bonds with the ion, resulting in extremely strong binding.

2.2 The constituents of commercial trypsin and the trypsin/EDTA solution used routinely in cell culture

Commercially available trypsin is generally prepared from bovine pancreas, the three main types being: crude trypsin powder, purified trypsin powder and crystalline trypsin.

Crude trypsin is most damaging to the cell and is mainly used for tissue disaggregation where its aggressive action is necessary. Batches can vary significantly in composition and therefore in the activities of the components; the enzyme activity may even depend on the activities of enzymes other than trypsin (Pine *et al*, 1969a; 1969b): one batch was found to kill over 90% of cells treated at 37°C for 15 minutes (Litwin, 1973). Hebb and Chu (1959) found marked differences in the degree of alteration of cell morphology following trypsinization with different serum batches; this was thought to be due to variations in their trypsin inhibitor activity (Stansly and Ramsey, 1957).

Purified trypsin powder contains only traces of such contaminating enzymes, and crystalline trypsin even less so are more consistent in

their constituents; it is these which are used to trypsinize continuous cell lines, and when sterile, may be kept frozen for 3 to 6 months at -18^{o}C without loss of activity (Montes de Oca, 1973).

The type of trypsin used, the lot of trypsin and the lot of serum used may therefore be an important factor to consider when interpreting results. The earlier studies of the effects of trypsin on the cell involved much more aggressive use of trypsin than is presently used to routinely passage cells in culture; this must be borne in mind when interpreting results. Considerable variation is also seen in the trypsinization solution used for similar purposes for example, Lamb and Ogden (1987) used 4 times higher concentration of EDTA and 5 times higher concentration of trypsin than used in this investigation (see Table 1.1).

3. The interaction of trypsin and EDTA with the cell surface and cell permeabilization

3.1 The action of trypsin on the cell surface and membrane

Gross morphological changes of cells are observed when cells are detached by trypsin, changing in shape from flat to round and contracted (Taylor, 1961; Paul, 1975), having to almost halve their surface area at constant volume (Burrows and Lamb, 1962) and acquiring the morphology of mitotic cells (Reddy *et al*, 1989) - compare plates 1.1 and 1.2. This effect is reversible however; cells will begin to reattach and flatten within one hour of resuspension in medium and incubation, attachment generally being complete after 2-3 hours. This reattachment involves a membrane molecule with two attachment sites (Heath, 1986; Horowitz *et al*, 1986); one to attach to the extracellular matrix, and another for talin, a cytoskeletal protein which is found at sites where

Table 1.1 Published protocols for trypsinization of cultured cells

Reference	Cell line	Trypsin	EDTA	Other details
Bender and Gooch, 1962	HeLa S3	0.5g/l	-	10 min
	GH7A6 human heart muscle	0.5g/l	-	10 min
Phillips and Tolmach, 1964	HeLa	0.3g/l	-	5 min at 38°C
Berry *et al*, 1966	V-79	1g/l	Ca^{++} and Mg^{++} free	8 min in saline
	HeLa	2.5g/l	-	in 3% sodium citrate in saline
Raaphorst *et al*, 1979	CHO	1g/l	-	in McCoy's 5a medium and 10% calf serum
Bryant and Parker, 1979	HeLa	0.5g/l	-	-
Sun *et al*, 1986	ESH5	0.5g/l	0.2g/l	-
	HeLa/human skin fibroblast hybrid	0.5g/l	0.2g/l	-
Lamb and Ogden, 1987	HeLa	2.5g/l	-	5 min
Reddy *et al*, 1989	V-79	0.5g/l	0.2g/l	10 min at 37°C
This thesis	CHO	0.5g/l	0.2g/l	10 min at 37°C
	EAT	0.5g/l	0.2g/l	10 min at 37°C

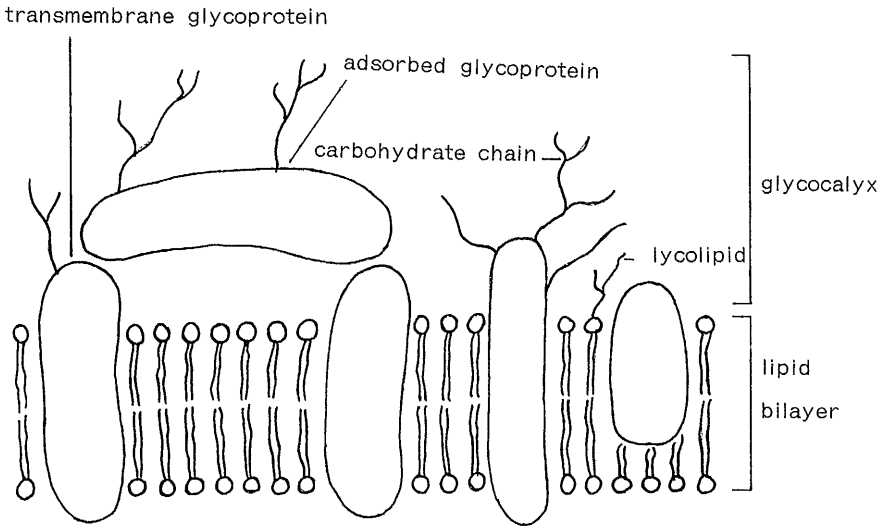


Figure 1.1 Schematic diagram of the glycocalyx (cell coat) and cell membrane

actin filaments connect with the cell membrane. Actin must reassemble its part of the cellular cytoskeleton after trypsinization so that the cell can flatten onto the substrate again. No further morphological changes to mitotic cells were noted after trypsinization (Reddy *et al*, 1989).

Trypsin is thought to detach cells by removing part of the cell coat involved in cell adhesion (Cook *et al*, 1960; Uhlenbruck, 1961; Laws and Strickland, 1961), and the detachment of these molecules is studied to gain information on cell surface structure (Rasilo and Yamagata, 1989). A schematic diagram of the cell membrane and cell coat is shown in Figure 1.1. The cell surface components are left behind on the flask (Weiss and Coombs, 1963) and appear to make it easier for cells to reattach to the same flask (Weiss, 1961). The trypsin stays attached to the cell, remaining active (Poste, 1971) and preventing the short-term

reformation of the glycoprotein cell coat material involved in cell attachment. The trypsin is gradually lost from the cell surface over the following 24 hours. Cell attachment in medium is not affected by adding 0.01% trypsin to the medium, varying the pH from 5.8 to 8.9 or adding 1% versene to the medium (Taylor, 1961). The ease with which cells may be trypsinized off a surface has been used to identify defects in the way in which the cell membrane interacts with the extracellular matrix (Simon *et al*, 1989; Kent, 1983).

Cell membranes are permeabilised much more and cell lysis is more common when using crude trypsin than crystalline, but the amount of membrane protein released appears similar for both preparations (Snow and Allen, 1970).

3.2 The entry of trypsin into the cell and cell permeabilization

Trypsin is known to transiently increase cell permeability (Levine, 1960), the cell recovering within 15 minutes of trypsinization (Lamb and Ogden, 1987); and it is thought that prior trypsinization aids the entry of other molecules such as REs into the cell (Obe and Winkel, 1985) when studying the way in which such enzymes cut ds DNA *in vivo*, for example, to investigate their role in the induction of chromosomal aberrations (Obe *et al*, 1985). Trypsinization is not an essential step as other techniques using non-trypsinized cells are also successful (Bryant, 1984; Bryant and Christie, 1989) even though Lamb and Ogden (1987) found no increase in permeability after trypsinization of cells already in suspension.

Lamb and Ogden (1987) postulated that the trypsinization process itself caused the cells to become more permeable, the gross rearrangements of the cell membrane required to break the connections with the surface to which cells are attached and the large reduction of surface area possibly leading to transient cell leakiness itself. This

increase in permeability is thought to be the reason for the release of low molecular weight components from cells after trypsinization (Hebb and Chu, 1969; Levine, 1960). Hodges *et al* (1973) showed that when cells are treated with trypsin the enzyme is not only bound to the cell surface but is also taken up into the cell; the presence of the enzyme in the cytoplasm, nucleus and nucleolus was seen to persist for up to 48 hours after trypsinization. It was not proven however whether the trypsin was still active within the cell and so it is not known whether it could be acting on essential components within the cell as well as the membrane.

Schor (1979) showed that trypsin and EGTA (binds Ca^{++} to the same extent as EDTA but binds Mg^{++} much less) remove different quantities and types of molecules from cell surfaces so it is likely that the same is true for EDTA: EDTA has been shown to remove peripheral proteins from cell membranes. In addition, as intercellular bonds are mediated by cations, especially Ca^{++}, the chelation of these ions may affect cell-cell interactions.

4. The interaction of trypsin/EDTA with chromatin

DNA has no one fixed conformation but is in a permanent metastable state, demonstrating a continuous cycle of change, controlled by subtle variations in its environment. The different states must be considered if perturbations of the DNA structure by trypsin are to be interpreted correctly. The diverse range of chromatin conformations are all produced by varying degrees of folding of the DNA double helix. These levels are illustrated in Figure 1.2. This section considers possible interactions of trypsin with chromatin over this wide range of folding.

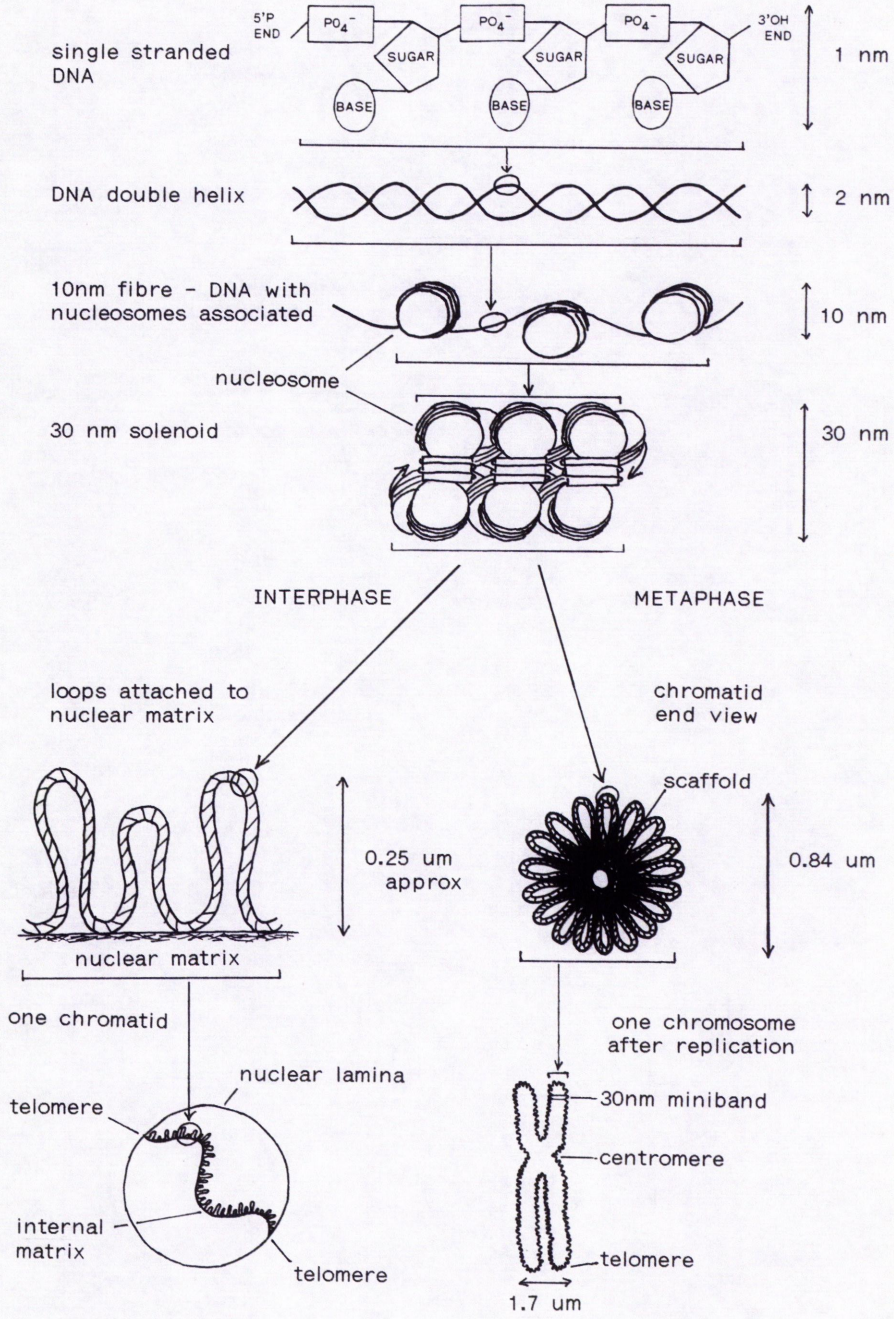


Figure 1.2 Schematic diagram of some current models of the levels of organisation of chromatin

4.1 The DNA double helix

Trypsin has a very specific action, only cleaving amino acids at lysine and arginine (i.e basic) residues and so would not attack DNA *per se* but could act on other (protein) components in chromatin. A complete digestion of interphase chromatin with trypsin destroys the *in vivo* structure but leaves a fine fibre which is thought to be pure ds DNA (Du Praw, 1965a).

EDTA also would not interact with pure DNA directly, only chelating Mg^{++} and Ca^{++}.

4.2 Higher order structure of Chromatin

Double stranded (ds) DNA does not exist free in the cell. At the first level of organisation the ds DNA is wrapped around core particles of histone octamers (two copies each of H2A, H2B, H3 and H4) to form nucleosomes, many of which on the DNA give a beaded chain or 10 nm fibre (Sedat and Manuelidis, 1977; Thoma *et al*, 1979). DNA associated with histones is known as chromatin. These histones have high proportions of basic amino acids (lysine and arginine) and so are attracted to the acidic (negative) DNA; the positive charges help to shield the negatively charged adjacent phosphate groups on the DNA, enabling it to fold up. These basic amino acids could be a target for trypsin if it were active within the cell, especially the linker histones (H1) and the core histone 'tails', both of which are highly basic. The action of trypsin on histone H1 has been published (Kohl), and trypsin will detach the core histone tails uniquely from H1-depleted chromatin *in vitro* (Bohm *et al*, 1980; 1981). After tryptic removal of core histone tails from the 10nm fibre, the number of initiation sites for E-coli RNA polymerase is doubled (Allen *et al*, 1982), presumably due to

the unfolded state of the chromatin but there is no evidence yet for this occurring *in vivo*.

Any further packaging of the DNA varies depending on the phase of the cell cycle, ionic environment and whether all the required proteins are present. Histone H1 (or a variant known as H5 in erythrocytes) 'sits' on the nucleosome where the DNA enters and leaves it, and along with other non-histone proteins enables the 10nm fibre to fold up into the solenoid structure known as the 30nm fibre; this is thought to be one type of *in vivo* folding (Davies *et al*, 1974; Ris and Kubai, 1970; Labhart *et al*, 1982) but its exact structure has not yet been elucidated, although is most likely to be solenoidal (Woodcock *et al*, 1984; Finch and Klug, 1976). Polynucleosomes with core histones without tails remain unfolded under any conditions, and when the tails are replaced artificially with basic polypeptides in the presence of H1, the 30 nm fibre will reform (Allen *et al*, 1982). Further packaging of the 30nm fibre is still controversial, the radial loop model thought to be the most appropriate theory to date for chromatid structure (Paulson and Laemmli, 1977; Earnshaw and Laemmli, 1983).

In interphase, transitions between different levels of packaging coincide with varying template activity on the chromatin. Inactive chromatin (heterochromatin) is tightly packed, or condensed, presumably preventing the RNA polymerase from entering the structure and large such areas are seen in the nucleus where none of the genes are active. The less condensed structure of active genes (euchromatin) may be clearly seen in the lampbrush chromosomes of amphibians and the polytene chromosomes of insects (Hill *et al*, 1987) as the chromosomes are so large. In a typical eucaryotic cell, only 5-10% of the genome is transcribed at any one time (Davidson, 1976). If trypsin did cause locally relaxed packing of chromatin *in vivo* then increased expression of a gene than normal could produce harmful effects on the cell, and if the

DNA is more extended it would be less protected and so more susceptible to X-ray damage. Partial trypsin digestion of chromatin *in vitro* results in the unfolding of the interphase fibres and the 'springing out' of the DNA (Du Praw, 1956b).

None of the above effects of trypsin have been investigated after routine trypsinization. A recent idea is that chromatin contraction occurs after trypsinization (Reddy *et al*, 1989) similar to that induced by hypertonic treatment, the chromatin condensing down in a manner more typical of metaphase chromosomes (Dettor *et al*, 1972).

Alternatively, trypsin could act on important (*eg* repair) enzymes in the cell, impairing their function.

If EDTA also entered the cell at trypsinization then it could also interact with intracellular components; EDTA has been shown to form stable complexes with Ca^{++} and Mg^{++} in deoxyribonucleoproteins, disrupting their structural stability (Mazia, 1954) and has also been shown to damage mitochondria (Harris and Leone, 1966).

4.3 DNA and the nuclear matrix

DNA does not exist as a pure and isolated molecule within the cell but has a highly complex configuration in close association with other structures (see Figure 1.2). Eukaryotic cell nuclei are thought to contain a complex 3-dimensional fibrogranular 'network' first named the nuclear matrix by Berezney and Coffey (1974) and is the term generally used (Capco *et al*, 1982; Fey *et al*, 1984), but also known as, for example, the nucleoskeleton, nuclear skeleton, karyoskeleton (Franke, 1987). The nuclear matrix is thought to represent 10-20% of the total nuclear protein (Berezney and Coffey, 1975). For reviews see Berezney and Coffey, 1976; Agutter and Richardson, 1980. Chromatin is thought to exist in close association with this matrix (Cook and Brazell, 1975, 1976). The nuclear matrix consists of 3 distinct parts: firstly the

peripheral outer region known as the nuclear lamina including pore complexes - the inner region of the nuclear envelope; secondly the internal matrix, the 3 dimensional network within and attached to the nuclear lamina (an alternative, equally correct, definition is for the internal matrix to be termed simply the 'nuclear matrix', but this can lead to confusion when discussing the internal matrix and lamina system as a whole); thirdly a residual nucleolar structure (Franke *et al*, 1981).

4.3.1 The nuclear lamina

The nucleus is bounded by the nuclear envelope, which consists of 3 layers: the outer membrane, the inner membrane and the nuclear lamina. Nuclear pores connect all 3 layers and facilitate the movement of small molecules across the nuclear envelope (Gerace, 1985). The nuclear lamina (with pore complexes) is part of both the nuclear envelope and the nuclear matrix and has been well characterised (Gerace and Blobel, 1980). The nuclear lamina consists of 3 major polypeptides: lamins A, B, and C with Mr 60,000-70,000 (Aaronson and Blobel, 1975; Gerace *et al*, 1978; Gerace and Blobel, 1980; Jost and Johnson, 1981; Gerace *et al*, 1984; reviewed by Krohne and Benavente, 1986; McKeon, 1987) which are thought to be another type of intermediate filament (McKeon *et al*, 1986; Franke, 1987). The structure of the lamina is independent of RNA. Certain regions of the lamina bind to specific proteins at the inner nuclear membrane and other regions bind to specific sites on the chromatin including the telomeres (Herrmann, 1989), playing a major part in its organization. The heterochromatic parts (*ie* telomeres and centromeres) of *Drosophila* salivary gland chromosomes are in stable contact with the nuclear envelope (Hochstrasser *et al*, 1986); the centromeres are grouped together at one region of the nuclear envelope and telomeres at the opposite side (Sedat and Manelidas, 1978).

The lamina is thought to be important in the breakdown and reassembly of the nuclear envelope at mitosis (Jost and Johnson, 1981). During prophase, DNA contacts with the lamina are lost, but are regained during telophase (Jost and Johnson, 1981).

The lamina is known to be extremely flexible and very sensitive to cellular perturbation.

4.3.2 The residual nucleolar structure

The nucleolus contains DNA from the nucleolar organizer (located at the tips of 5 different chromosomes), granular mature ribosomal precursor particles and a dense fibrillar component, which consists of RNA transcripts. There is no membrane enclosing the structure which instead appears to be maintained by the association of ribosome precursors by unknown means. The nucleolus is also attached to the matrix and shows a residual structure on matrix preparation (Franke *et al*, 1981).

4.3.3 The internal matrix

Some argument still surrounds the existence of an internal matrix, thought to be a 3-dimensional structure analogous to the cytoskeleton. As the matrix has not yet been observed *in vivo*, some form of isolation is always a prerequisite for its study; as nuclei and chromatin aggregate at around isotonic NaCl concentrations the preparation procedure of matrices has always involved extreme non-physiological conditions (nuclease digestion, high salt extraction and non-ionic detergent) - the validity of using such procedures to prepare matrix structures in the same form as exists *in vivo* has produced much controversy. The differences in matrix-like structures isolated using similar techniques (compare Berezney and Coffey, 1974 and Aaronson and Blobel, 1975; also see Skaer and Whytock (1977); Kaufmann *et al*, 1981; Miller, 1984; Bekers

et al, 1986) have prompted the suggestion that the internal matrix is simply an artefact of preparation procedures and the removal of the DNA as part of the nuclear matrix preparation procedure is thought to cause rearrangements resulting in major artefacts being observed (Gallinaro *et al*, 1983). Even though the matrix may be clearly studied in isolation it may not therefore be demonstrating the true *in vivo* structure.

More recent results have, however, provided more evidence for the existence of the nuclear matrix, but total agreement has still not been reached over this matter. The discrepancies in nuclear matrix preparation (Berezney and Coffey, 1974 and Aaronson and Blobel, 1975) have since been explained (Kaufmann *et al*, 1981; Berezney and Coffey, 1977; Lebkowski and Laemmli, 1982); recent electron microscopy evidence of an internal matrix in resinless sections of chromatin-depleted nuclei (Brasch, 1982; Guatelli *et al*, 1982; Capco *et al*, 1982; Fey *et al*, 1984) show an internal matrix structure made up of RNA particulate structures interspersed between longer protein filaments, thought to consist at least partly of lamins but other proteins have also been identified (Verheijen *et al*, 1986) including more specialized proteins such as DNA polymerase (Smith and Berezney, 1980), RNA polymerase II (Lewis *et al*, 1984) and topoisomerase II (Gasser *et al*, 1986; Vassetzky *et al*, 1989). The protein composition is thought to be differentiation-stage dependent (Stuurman *et al*, 1989) and cell cycle phase dependent (Hodge *et al*, 1977). The structure of the inner matrix is disrupted if the RNA is removed under conditions where S-S cross-linking is prevented (Kaufmann *et al*, 1981), inferring that RNA forms an integral part of the inner matrix structure. Chaly *et al* (1984) have also reported the production of antibodies to non-lamin components of the matrix. Some kind of ribonucleoprotein matrix is thought by many to be present in all eukaryotes, but as the matrix has not yet been observed *in vivo*, various methods of isolation have been used to obtain residual nuclear

matrix structures. These are classified and named according to the method and degree of removal of the *in vivo* structure, including nuclear matrix (Berezney and Coffey, 1974; Agutter and Richardson, 1980), ghost (Keller and Riley, 1976; Agutter and Richardson, 1980) and cage (Cook and Brazell, 1978). 'Nuclear matrix' refers to both the *in vivo* and *in vitro* (nuclease digestion, high salt extraction and non-ionic detergents) structures which are clearly very different. It is important to know which of these are being discussed as this difference is not always very clear in the literature.

The matrix is stabilized by metalloprotein interactions (Lebkowski and Laemmli, 1982), possibly Mg^{++} or Cu^{++}. If EDTA entered the cell, it could possibly disrupt this balance, although EDTA treatment of isolated matrices had no affect unlike other chelating agents.

4.4 Interphase chromosome structure

Chromatin is thought to be attached to the nucleoskeleton in many supercoiled loops (Cook and Brazell, 1975; 1976; Feinberg and Coffey, 1982). The use of nucleoids to study this will be discussed in detail in Chapter 6. Briefly, there are thought to be 2 levels of folding of the DNA within the nucleus. Firstly, the DNA is attached in large structural loops to the nuclear lamina. Secondly, the DNA interacts with the internal matrix (Lebkowski and Laemmli, 1982a, 1982b; Lewis *et al*, 1984). Wanka *et al*, (1982) suggested that nuclear DNA is attached to 3 elements of the nuclear matrix: lamina, pore complexes and internal matrix. Many internal matrix structures have been shown to be DNA binding proteins (Comings and Wallach, 1978). Current theory suggests that it is the expressed regions of genes that are in transient association with the matrix (*eg* Ciejek *et al*, 1983; Robinson *et al*, 1982; Cook *et al*, 1982).

The nuclear matrix is now thought to play an active functional role within the cell as well as structural (Vogelstein *et al*, 1980). After

nuclease treatment of cells which removes most of the chromatin, a residual nucleoskeleton has been shown using various matrix isolation methods to act as a skeleton at which DNA replication (Maul, 1977; Dijkwel *et al*, 1979; McCready *et al*, 1980; Vogelstein *et al*, 1980; Pardoll *et al*, 1980; Berezney and Buchholtz, 1981; Berezney *et al*, 1982; Wanka *et al*, 1982; Jackson and Cook, 1986) and transcription (Jackson *et al*, 1981; Robinson *et al*, 1982; Ross *et al*, 1982; Ciejek *et al*, 1983 Jackson and Cook, 1985; Fisher *et al*, 1989) can occur (discussed by Razin, 1987). Transcribed genes lie close to this residual structure (Jackson *et al*, 1981) and nearly all nascent RNA is also associated with the structure (Jackson *et al*, 1981; Capco *et al*, 1982; Long *et al*, 1979), both ends of transcripts being attached (Jackson *et al*, 1981). Only mature RNAs are removed from the nucleus by high salt extraction (Ciejek, 1982; Ross, 1982) which is thought to be due to changes in the state of attachment (Denome *et al*, 1989). This residual nucleoskeleton also provides binding sites for hormones (Barrack and Coffey, 1980) and has been shown to be a site of RNA processing (Ciejek *et al*, 1982). Viral genomes are also transcribed and replicated in association with the nucleoskeleton preparation during infection (Cook *et al*, 1982; Jackson *et al*, 1982; Ben-Ze'ev *et al*, 1982; Smith *et al*, 1985); Chai and Sandberg (1988) reported that the nuclear matrix formed boundaries round each interphase chromosome, resulting in channels connecting nuclear pores and nucleoli.

Even though recent evidence favours the existence of an internal matrix, it must be remembered that there is as yet no unanimous agreement on this (discussed by Cook, 1988).

4.5 Metaphase chromosome structure

In mammalian cells, an electron microscope is required to pick out differences in interphase structure, which has the same diffuse structure under the light microscope. During mitosis, however, the

chromatin structure condenses in a very ordered way to form mitotic chromosomes which are clearly visible under a light microscope.

The metaphase structure thought to organize the chromatin structure into loops has been named the scaffolding (Paulson and Laemmli, 1977; Adolph *et al*, 1977a; 1977b; Laemmli *et al*, 1978; Adolph, 1980), consisting of several non-histone proteins (far fewer than in the metaphase matrix), including 2 prominent proteins of high molecular weight (Sc_1 and Sc_2, Mr 170,000 and 135,000 daltons, Lewis and Laemmli, 1982) which make up at least 40% of the metaphase scaffold and are also thought to be (minor) components of the interphase internal matrix (Vassetsky *et al*, 1989). Sc_1 is now thought to be topoisomerase II (Earnshaw *et al*, 1985) and is thought to be involved with the regulation of supercoiling of individual domains. The scaffold may be isolated independently of the chromatin (Adolph *et al*, 1977). RNase treatment was not found to affect the scaffolding structure (Adolph *et al*, 1977) either because RNA was not fundamental to the structure (unlike in the interphase nuclear matrix) or because the structural RNA is protected by proteins. The scaffold is also thought to be stabilized by metalloprotein interactions (Lewis and Laemmli, 1982); again, EDTA could interact with these causing destabilization. The genome in metaphase chromosomes has been shown to be organized into supercoiled loops anchored at their bases (Paulson and Laemmli, 1977; Adolph *et al*, 1977a; 1977b), where the structure when depleted of histones and most non-histone proteins retains various morphological features of metaphase chromosomes (Paulson and Laemmli, 1977; Earnshaw and Laemmli, 1983). The 30nm fibre is first thought to be folded up into large, looped domains of approximately 400nm length, anchored at their bases by DNA binding proteins which recognise certain DNA sequences. This 'string' of loops is then further coiled radially to produce a highly condensed chromatid of 840nm diameter with a central axis, 2 together making up a metaphase

chromosome (Pienta and Coffey, 1984). As for the nuclear matrix, the structure of the scaffold is retained after DNase digestion of the DNA.

The basis of G-banding is to remove nucleoproteins from fixed chromosomes by proteolytic enzymes such as trypsin (Wang and Fedoroff, 1972), resulting in localized 'despiralization' or expansion of regions of the chromosomes. The bands can be seen even before staining with phase contrast Nomarski optics as they appear as elevations on the chromosome surface. The trypsin is thought to relax regions of the packed chromatin fibre, possibly reflecting differences in chromatin packing in different regions of chromatids (proposed by Du Praw, 1965a; 1965b) but the true physicochemical nature of the effect is not yet understood. The trypsin-treated chromosomes are seen to have variable surface structure in different metaphase spreads on the same slide, some still appearing smooth and compact while others appearing filamentous and knobbly which may be due to variations in the degree of chromosome condensation between cells, or due to varying amounts of protective cytoplasm and/or cell membrane remaining on the slide. Giemsa does not bind well to these nucleoprotein-depleted regions of the DNA and so these appear pale. The accumulation of Giemsa between the coils in the chromatid arms is thought to cause G-band staining .

Trypsinization has been shown to increase the number of chromosomal abnormalities (Levan and Biesele, 1958), but by using a more aggressive treatment than that used for routine trypsinization.

5. The potentiation of X-ray damage by trypsin

Most studies of the trypsin effect to date have investigated the effect of trypsin on X-ray induced cell killing using a clonogenic assay to obtain survival curves; for example, see figure 1.3. The results of these studies will be discussed in detail in Chapter 3. Briefly, all published work on a range

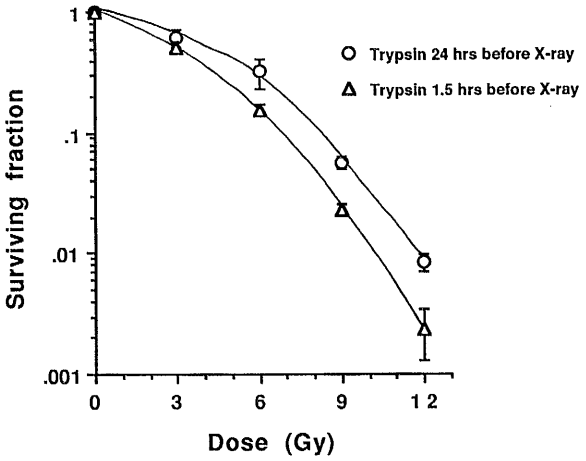


figure 1.3

Evidence for the trypsin effect in V-79 Chinese Hamster cells obtained using survival curves. Data from Sprunt, 1986.

of cell lines showed that trypsinization prior to irradiation resulted in a survivial curve with decreased D_o (Barendsen and Walter, 1964; Berry *et al*, 1966; Lehnert, 1975; Bryant and Parker, 1979; Reddy *et al*, 1989), although the change in D_q after irradiation varied between cell lines, both increases and decreases having been reported by the above authors.

Interpretations of the trypsin effect are generally made at the cellular level and various explanations have been suggested as will be discussed in Chapter 3. Ideas include trypsin-induced cell synchrony and the accumulation of cells in more radiation-resistant cell cycle phases (*eg* Berry *et al*, 1966), reduction of the plating efficiency of irradiated cells by the interaction of trypsin with radiation damage to the cell membrane (Sun *et al*, 1986) and cell attachment mechanisms (Bender and Gooch, 1962), and a reduction in repair capacity of cells rounded up after trypsinization (Elkind *et al*, 1961; Reddy *et al*, 1989).

At the chromatin level, it is thought that when a cell is killed by ionizing radiation this is due to the radiation damaging a unique sensitive target within the cell. It is possible that trypsin may interact with this target to make it more sensitive. Evidence suggests that cell death after X-irradiation occurs when the genetic apparatus (*ie* chromatin, DNA) suffers some kind of irrepairable damage or undergoes 'mis-repair'. It is therefore also possible that trypsin potentiates X-ray damage by affecting the genetic material in some way.

An unrepaired dsb may lead to the death of the cell (Blocher and Pohlit, 1982) or give rise to a chromosomal aberration which can kill the cell (Bryant, 1985) so trypsin could potentiate X-ray damage by increasing the incidence of dsb or by reducing the rate of dsb repair; prior trypsinization has already been shown to reduce the efficiency of excision repair (ssb repair) after UV irradiation (Collins, 1987; Kaufmann and Briley, 1987). Moreover, chromosomal aberrations (CA) are thought

to result from DNA dsb, so if trypsin acted in this way, an increased frequency of CA may be seen if cells were trypsinized before irradiation.

These ideas will be expanded upon in the relevant chapters, including discussion of what is known about DNA damage and repair at the different levels of cellular organization.

6. Aims of the investigation

Trypsinization of cultured cells is employed in virtually all assays of cellular, cytogenetic and DNA damage resulting from exposure of cells to radiation or other genotoxic agents. Radiation damage can be mimicked by treatment of permeabilized cells with restriction endonucleases (RE) (Bryant, 1984; 1985) and techniques involving prior trypsinization of cells have been used to permeabilize cells to RE (Obe and Winkel, 1985); this may also result in the entry of trypsin into cells. If the trypsin effect were due to the interaction of trypsin with intracellular proteins *eg* the histones or non-histone proteins associated with DNA, this could potentiate the effect of the RE by uncovering restriction sites.

The purpose of this study was to investigate the underlying mechanisms of the trypsin effect, using various end points to determine the influence of trypsin on X-ray induced cytogenetic and DNA damage, and on the capacity of cells to repair DNA damage.

Firstly, the effect of trypsin in combination with X-rays on two cell lines was investigated using clonogenic assays. No published reports could be found relating to studies of the trypsin effect in cells growing entirely in suspension so the effects of trypsin on X-irradiated suspension cells was studied here. Secondly, chromosome damage was scored as anaphase chromosome abnormalities in X-irradiated and

trypsinized cells to determine whether trypsinization also increased the frequency of chromosome abnormalities. Thirdly, evidence was sought for chromatin being a sensitive target for trypsin within the cell by using two methods of measuring X-ray induced strand breakage: DNA unwinding and neutral elution.

In an attempt to measure restriction endonuclease and trypsin damage in chromatin a technique was developed which proved to be interesting in itself both as a method to measure DNA damage but also in yielding information about nucleoid formation and structure.

This account is structured in such a way that each chapter describes the investigation of the trypsin effect assayed using a different end-point; the techniques used and the reasons for their selection will be discussed fully in the relevant chapters. The final chapter discusses the results in relation to each other and to other published findings.

Chapter 2

GENERAL MATERIALS AND METHODS COMMON TO ALL EXPERIMENTS

1. Cell culture

2. Preparation of a suspension of CHO cells which grow attached to culture flask surfaces

3. Trypsinization of EAT cells

4. Preparation of stationary EAT cells

5. X-irradiation

Chapter 2

1. Cell Culture

Two cell lines were used in this investigation: a Chinese Hamster Ovary line (CHO K1) and Erlich ascites tumour cells (EAT) - a mouse line.

CHO K1 cells were used for much of the original chromosome aberration work as this cell line has a relatively small number of comparatively large chromosomes (modal chromosome number is around the true diploid number of 2n = 22) and so they tend to give good chromosome preparations and a relatively high proportion of metaphases. The line is fibroblastic and cells grow attached to the culture flask with a doubling time of 12-14 hours; in addition, they give rise to small, tight colonies and so are useful for clonogenic assay studies. CHO cells were used here for clonogenic assays so that the effect of trypsin could be compared with existing results for V79 cells - another Chinese hamster line. CHO cells were routinely passaged in $75cm^2$ tissue culture flasks (Sterilin) in Eagle's minimal essential medium (MEM) supplemented with 15% foetal calf serum (FCS), non-essential amino acids, L-glutamine and antibiotics (penicillin and streptomycin) and incubated at 37^oC in air containing 5% CO_2.

EAT cells can produce ascites tumours in the abdomen of mice as well as solid tumours in their legs. They may be grown *in vitro* either in suspension when the growth medium has a low Ca^{++} concentration ('A2' medium was used: Iliakis and Pohlit, 1979) or attached to the culture flask surface when in high Ca^{++} concentration (MEM medium was used). In this investigation EAT cells were grown in suspension and passaged daily in A2 medium from $8x10^5$ cells/ml to $2x10^5$ cells/ml to keep the cells in exponential phase, pipetting vigorously at each passage

to keep the cells in suspension. The cells also are stable in stationary phase, a property used to induce cell synchrony. They are also a good line to use in DNA unwinding experiments as their repair has been well characterised using this technique. A relatively high proportion of anaphases are also seen in EAT cell populations which may be greatly increased by synchronization,so are useful to use in anaphase aberration assays. Problems were previously encountered when attempting to devise a valid non-trypsinized control for attached cell cultures (Sprunt, 1986) so another advantage of using EAT cells in this investigation is that because they grow in suspension, it was not necessary to trypsinize them for experiments and a comparison could be made between trypsinized and non-trypsinized cells without the need for scraping to prepare (non-trypsinized) controls; the cells could be treated instead with the EDTA/buffer solution in which the trypsin is dissolved. This solution is referred to as 'buffer' in the text.

It was important to grow the EAT cells in non tissue culture Petri dishes or in $75cm^3$ flasks stood on end so the cells were only in contact with a limited area of the bottom face of the flask as this was treated to encourage cells to attach. EAT cells were incubated at $37^{o}C$ in 6% CO_2. The cells as first defrosted showed a tendency to clump and to attach to the sides of the container. To improve the suspension, at each daily passage the cells were pipetted into 10ml 'U' centrifuge tubes and spun for 30 seconds at 1000rpm. The supernatant was then removed, leaving behind the large clumps of cells which had been spun to the bottom of the tube. The cells in the supernatant were counted, diluted in fresh medium and grown up in fresh flasks. Cells were found to attach less well to fresh flasks than to those in which they had been growing for a few days. This procedure was repeated until a consistent cell suspention could be obtained at each passage by just pipetting; this took some weeks for most defrosted samples.

2. Preparation of a suspension of CHO cells which grow attached to culture flask surfaces

The routine preparation of a cell suspension is an essential part of the passaging procedure; as the techniques used were fundamental to this investigation they will be considered in detail. CHO cells grow as a monolayer attached to the surface of the flask and were trypsinized from flask surfaces as part of the routine passage procedure - 2 washes with a standard trypsin/ethylene diamine tetra-acetic acid (EDTA) solution: 0.05% trypsin (Bacto Trypsin Difco), 0.2g/l EDTA in buffer/salts solution (8g/l NaCl, 0.2g/l KCl, 1.15g/l Na_2HPO_4, 0.2g/l KH_2PO_4), incubated at 37°C for 5 minutes then resuspended in MEM to stop the trypsin digestion. The suspension was then pipetted to separate any clumps.

3. Trypsinization of EAT cells

For trypsin or buffer treatment of EAT cells, samples of cells were dispensed into 10ml sterile 'V' centrifuge tubes and pelleted (5 minutes at 1000rpm). The cells were not pelleted any more vigorously as this was found to cause the trypsinized samples especially to clump. The medium was aspirated and the pellet loosened by gently vortexing, then 5ml of either trypsin or buffer was added to each tube and incubated for 5, 10, or 15 minutes at 37°C. For the major part of this time the cells were incubated at 37°C but in the last 3 minutes the cells were centrifuged at 1000rpm at room temperature, so that exactly at the end of the incubation time the solution could be drawn off and the cells resuspended in fresh growth medium, pipetting well to disperse any clumps.

4. Preparation of stationary EAT cells

Another useful characteristic of EAT cells is that if they are not passaged daily they will soon reach stationary phase, in which they are stable for several days. Exponentially growing cells were diluted to $2x10^4$ cells/ml in fresh A2 medium and dispensed into non-tissue culture Petri dishes. The cells were then incubated for 4 days by which time the cell concentration had reached $2x10^6$/ml and the population had become stationary. To bring the cells back into cycle, they were spun out of the conditioned medium and resuspended in fresh medium at a concentration of $2x10^5$ cells/ml. The cells should all come back into cycle at the same time and as they were all stationary in G_0 they should be synchronous.

5. X-irradiation

Cells were exposed to X-rays (250kV, 14mA, 0.5mm Cu filter) from a Siemens therapy unit giving a dose rate of 0.75 Gy/min or 5.8 Gy/min and were contained either in $25cm^3$ plastic flasks or 7ml plastic bijou bottles. Doses were checked by a ferrous sulphate method (Frankenberg, 1969).

Chapter 3

INVESTIGATION OF THE TRYPSIN EFFECT IN RELATION TO X-RAY INDUCED CELL DEATH USING A CLONOGENIC ASSAY

1. Introduction

   1.1 The potentiation of X-ray induced cell killing by trypsin
   1.2 Suggested explanations of the trypsin effect at the cellular level
   1.3 Aims of this chapter

2. Materials and Methods

3. Results

4. Discussion

Chapter 3

Here experiments are described where cell survival after trypsinization is investigated to verify the existence of a trypsin effect, including the EAT (suspension) cell line which has not previously been used to investigate this potentiation of cell killing.

1. Introduction

The main method used previously to investigate the trypsin effect was clonogenic assay, so it was necessary to confirm this trypsin effect in a cell line already known to show trypsin-induced potentiation of cell killing (CHO) and another in which the trypsin effect has not so far been investigated (EAT). For cultured cells, cell death is defined as the loss of reproductive integrity - a cell may be capable of a few divisions but cannot divide indefinitely and so cannot produce a colony. Ionizing radiation can induce this loss of reproductive integrity (Puck and Marcus, 1955), a property used directly to assay for radiation damage using clonogenic assay, a plot being drawn of the loss of reproductive integrity of the cells as a function of radiation dose, otherwise known as a survival curve. Clonogenic assay is a well established method in radiobiology as it is an end point which may be easily and accurately scored, information on cell killing and repair may be deduced from the resulting curves. This was one of the first techniques used to investigate the interaction of X-rays with the cell and is still a much used method. Cells are irradiated with a graded series of doses, then plated out at appropriate dilutions in Petri dishes and incubated until cell colonies form. The number of cells plated on each dish is known, so from the number of colonies on the dish the surviving fraction at each X-ray dose may be calculated (with correction for plating efficiency

deduced from the control sample colony count). A major use of this technique is to investigate PLD and SLD repair.

Ionizing radiation is thought to induce 3 types of damage into mammalian cells. Firstly, lethal damage (LD), which is irrepairable and inevitably leads to cell death. Secondly, sublethal damage (SLD: Elkind *et al,* 1961) which can normally be repaired unless the cell receives a second X-ray dose before the initial SLD may be repaired - this additional SLD may then interact with the existing SLD to result in LD. By definition, SLD must be accumulated in a number of critical sites before the damage becomes lethal, but can be repaired before this stage is reached. SLD is thought to be represented by the shoulder (*ie* D_q) in survival curves, and lethal damage by the exponential part (*ie* D_o). Split dose experiments are often used to study SLD, less cell kill being seen after a dose has been given in 2 fractions rather than as a single dose. This is thought to be due to the repair of SLD in the interval between doses. Lastly, potentially lethal damage (PLD: Phillips and Tolmach, 1966) which may either cause cell death or be repaired, depending on the postirradiation conditions. Cells incubated under suboptimal conditions after irradiation, for example in Hank's balanced salt solution or in density-inhibited stationary phase (Little and Hahn, 1973) showed a higher survival than those held in growth medium (Phillips and Tolmach, 1966; Belli and Shelton, 1969; Winans *et al,* 1972; Hahn *et al,* 1973; Sinclair and Morton, 1964; Little, 1973; Hetzel *et al,* 1976; Utsumi and Elkind, 1985) although Barendsen and Walter (1964) noted the reverse. There is evidence that PLD may reflect repair of dsb (Bryant, 1985; Frankenberg *et al,* 1984) but there is still some argument as to whether SLD and PLD represent the same lesion (Reddy and Lange, 1989) or whether they differ (Frankenberg *et al,* 1984).

1.1 The potentiation of X-ray induced cell killing by trypsin

Elkind *et al* (1961) were the first to suggest that trypsinization may be affecting the results of clonogenic assay as a result of studies of SLD, and it is now known that trypsinization immediately before irradiation increases the radiation sensitivity of mammalian cells (Barendsen and Walter, 1964; Phillips and Tolmach, 1966; Berry *et al*, 1966; Raaphorst *et al*, 1979; Djordjevic, 1979), radiosensitivity decreasing as the time between trypsinization and irradiation is increased and the effect generally being lost after 24 hours (Barendsen and Walter, 1964). A potentiation effect is also seen when cells are trypsinized after irradiation (Sun *et al*, 1986) and when trypsinizing cells which are being continually irradiated (Nias and Lajtha, 1964). The difference in survival curve parameters when using trypsinized and untrypsinized cells varied between cell lines; all published work and cell lines showed a decrease in D_o after trypsinization (Barendsen and Walter, 1964; Berry *et al*, 1966; Lehnert, 1975; Bryant and Parker, 1979; Reddy *et al*, 1989); regarding changes in D_q, authors using V-79 cells have shown a decrease after trypsinization (Lehnert, 1975; Reddy *et al*, 1989) while an increase was seen for T_1 human kidney cells (Barendsen and Walter, 1964) and HeLa (Bryant and Parker, 1979).

1.2 Suggested explanations of the trypsin effect at the cellular level.

Most observations of the trypsin effect were as a by-product of investigating SLD and PLD so interpretations are generally in terms of these. Split dose effects and the increased resistance to a second, later, X-ray dose, generally interpreted as being due to the expression of SLD, were shown by Bryant and Parker (1979) to be accounted for solely by the post-trypsinization increase in survival seen after any single X-ray exposure.

Sun *et al* (1986) thought that trypsinization was an important factor to consider when studying PLD repair as trypsinization soon after irradiation could fix PLD to LD. Reddy *et al* (1989) developed this idea still further by suggesting that differences in survival between immediate and delayed plating in growth medium was due only to the recovery from the trypsin effect; they added that pure PLD must be studied by comparing delayed plating either in growth medium or HBSS rather than trypsinizing and incubating for varying lengths of time in suboptimal conditions to eliminate effects due to the trypsin.

Various mechanisms have been suggested to explain pre- and post-irradiation trypsin effects. When trypsinizing before irradiation, Barendsen and Walter (1964) thought that the decrease in sensitivity with time between trypsinization and irradiation might be due simply to a recovery from the trypsinization procedure but it is generally thought to be more complicated than this. Although an overall decrease in sensitivity is seen as the time between trypsinization and irradiation is increased from 0 to 24 hours, the radiosensitivity fluctuates in a cyclic, repeatable way which many authors believe is due to trypsin causing partial synchrony in the cells (Berry *et al*, 1966; Lehnert, 1975; Raaphorst *et al*, 1979; Phillips and Tolmach, 1964) as the radiosensitivity of cultured mammalian cells is known to fluctuate throughout the cell cycle (Terasima and Tolmach, 1961; Sinclair and Morton, 1961; 1964; Belli and Shelton, 1969), although Raaphorst *et al* (1979) thought that the trypsin effect could not be entirely due to this. Barendsen and Walter (1964) saw a more progressive change in X-ray sensitivity and not a cyclic effect. They still thought that partial cell synchrony could play a part in the trypsin effect. The synchrony was thought to be induced by some form of cellular trauma connected with the trypsinization procedure such as the loss of cellular constituents (Puck *et al*, 1956) and is generally more apparent after higher doses than lower (Raaphorst

et al, 1979). Phillips and Tolmach (1964) showed that the cell cycle related peak in radiation resistance occurred at the time of maximum DNA synthesis (S phase) but in addition noted changes in the lengths of cell cycle phases after trypsinization, resulting in more cells in the more resistant phases; (Lehnert (1975) found the greatest surviving fraction in S phase and the smallest in G_2, M and G_1 for Chinese hamster cells). Berry *et al* (1966) later confirmed these effects. Collins (1987) found that cells began cycling several hours sooner in trypsinized cells than those scraped into suspension.

Sun *et al* (1986), trypsinizing after irradiation, suggested that trypsin could be interacting with radiation damage to the cell membrane and so reduce the plating efficiency of irradiated cells, their evidence being that far more cells are made permeable to the stain trypan blue after irradiation and trypsinization than after either treatment alone, taking this permeability to indicate membrane damage. This could then result in a change in intracellular ionic balance (known to occur after trypsinization), leading to possible changes in DNA conformation which could result in PLD fixation. Lehnert (1975), however, found no potentiating effect when trypsinizing immediately after irradiation. Bender and Gooch (1961) suggested that irradiation could damage the cell attachment mechanism of cells irradiated in suspension, the observed survival being reduced as unattached cells, even if still viable, would not form scorable colonies. It is feasible that trypsin could interact with this damage to increase the number of cells which cannot attach.

Reddy *et al* (1989) rejected such arguments as no dependence of the trypsin effect on the length of time of trypsinization or concentration of trypsin was found, which, they argued, should be seen if the trypsin effect is due to membrane damage. Barendsen and Walter (1964), however, did show a higher sensitivity to trypsin after longer

trypsinization times if irradiation was carried out immediately after trypsinization but no difference when irradiating after 8 hours.

From studies with V-79 cells, Reddy *et al* (1989) postulated that the trypsin effect was mainly caused by morphological changes of the cell induced by trypsin, altering the ability of the cell to repair radiation damage, having observed that spread cells were better able to repair damage than those in suspension. This is not a new idea: Elkind *et al* (1961) thought that the cyclic cell survival could be a reflection of shape changes as cells flatten during the first few hours after trypsinization.

Mitotic cells were not more sensitive to irradiation as a result of trypsinization: Reddy *et al* (1989) deduced from this that trypsin does not affect the survival of cells which are already round and show no change in morphology on trypsinization. Additionally, Reddy *et al* (1989) saw no difference in survival between cells trypsinized for 1.5 minutes (by which time they had rounded up) and 10 minutes (no further change in morphology). Cells trypsinized just before irradiation showed a lower survival than those trypsinized just after, as those cells trypsinized before irradiation will be rounded at irradiation and so more sensitive than those still attached to the culture flask. Cells trypsinized before irradiation only showed an increased resistance up to 2.5 hours between trypsinization and irradiation, after which time almost all cells had attached, whereas those maintained in suspension showed <u>no</u> change in survival as the time between trypsinization and irradiation was increased to 3-4 hours, then the survival decreased with time. Reddy *et al* (1989) thought this showed that cells do not recover from the trypsin effect as long as they remain round.

Little (1973) saw no PLD repair in stationary cells when incubating in growth medium or conditioned medium in suspension, but did see PLDR when the cells had attached and spread. However, Lehnert (1975)

saw no relation between radiosensitivity and attachment when studying the same cell line.

The existence of a trypsin effect when monitored by X-ray induced cell killing is clearly well reported. There is however as yet no unanimous agreement on the underlying mechanisms of the trypsin effect.

1.3 Aims of this chapter

As mentioned in the introduction, scraping was not found to be successful as a non-trypsin control and so clonogenic assay is the only successful method of investigating the trypsin effect in attached cells without having to scrape the cells off the dishes, as cells can be trypsinized, plated, then allowed time to recover from the trypsin effect before irradiation. This is therefore the only way of studying the trypsin effect due to routine passaging of attached fibroblasts; Berry *et al* (1966) compared the survival of HeLa cells trypsinized and shaken off the flask surface, and observed that the cells shaken off were more resistant to irradiation, although this was thought to be due to clumping of non-trypsinized cells and not the trypsin effect.

The CHO cell line was chosen as the trypsin effect has already been shown to be evident in this line (Berry *et al*, 1966). As mentioned in Chapter 2, it was decided to investigate the trypsin effect using a suspension culture to remove the necessity for detaching cells from dishes so the EAT cell line was also used here for clonogenic assay.

The aim of this investigation was to determine whether the trypsin effect due to routine trypsinization procedures would be an important effect to consider in experiments utilizing a variety of end points in which trypsinization is simply used to produce a cell suspension, so it was important here not to trypsinize immediately before irradiation but to leave a typical time interval for such an experiment. 45 minutes was

decided to be typical and so was used for this investigation. Lehnert (1975) observed a variable lag between trypsinization and the start of cell division which ranged from 2 to 6 hours in V-79 cells. This means that the cell multiplicity in different experiments where the cells are left for longer than 2 hours between trypsinization and irradiation will introduce greater uncertainty into experiments. Using a 45 minute time means that the cells should have gone back into cycle and these problems should be avoided.

To enable survival curves from CHO cells trypsinized at various times before irradiation to be compared, multiplicity corrections must be made. When the time interval between trypsinization and irradiation is long, the cells may have started to multiply so that at irradiation there may be more than one cell per colony. If just one of these cells is killed by the treatment, the colony will still survive and so the survival will be greater than if the cells were all single. To correct for this, extra cells are trypsinized and incubated at the same time as those in the experiment then fixed and stained when the rest of the cells are irradiated. The mean number of cells per colony (the multiplicity factor) is determined and the surviving fraction for each cell dose is divided by this multiplicity factor to obtain the points on the survival curve corrected for cell multiplicity.

Berry *et al* (1966) had noticed a cyclic change in X-ray sensitivity of CHO cells to trypsin as the time interval between trypsinization and irradiation was increased, which had since been confirmed in V79 cells (Sprunt, 1986) so this was to be investigated in the CHO line here over time intervals of up to one hour between trypsinization and irradiation, shorter than have been previously investigated.

2. Materials and Methods

Clonogenic assays for CHO cells were performed using standard procedures as shown in Figure 3.1. After treatment and appropriate dilution, cells were plated in 6cm plastic dishes in 5ml MEM and incubated for 6 days. Dishes were gently washed in pH 6.4 buffer then colonies were fixed for 10 minutes in methanol and stained in undiluted Giemsa for 10 minutes, gently rinsed in water and air dried.

EAT cells grow in suspension and so do not form colonies on dishes if plated out in the usual way, the technique used instead being shown in Figure 3.2. Cells were diluted, and samples of cell suspension mixed with double their volume of 2x concentrated A2 medium containing 0.6% agar (so the final concentration of agar was 0.4%) at 37^{o}C. This was poured into 6ml plastic dishes which already contained an underlayer of 0.5% agar in A2 medium. As the liquid agar cooled, it set and so immobilised the cells in the dish. When the cells divided, the daughter cells remained in the vicinity of the original parent cell and so colonies resulted. The dishes were incubated for 14 days, then left in air at room temperature overnight so that the soft agar overlayer solidified and provided a darker background against which the paler colonies could be scored.

3. Results

The survival curves of CHO cells trypsinized 45 minutes and 24 hours before irradiation lie quite close together (Figure 3.1(a)) so the potentiating effect due to trypsin was small (trypsinizing 45 minutes before irradiation: D_o=1.7 Gy, D_q=3.6 Gy; trypsinizing 24 hours before irradiation: D_o=1.9 Gy, D_q=4 Gy). For EAT cells trypsinized and EDTA treated 45 minutes before irradiation, a marginal separation was seen between the two sets of points (Figure 3.1(b)) but considering the uncertainties in each point a trypsin effect was not present and one

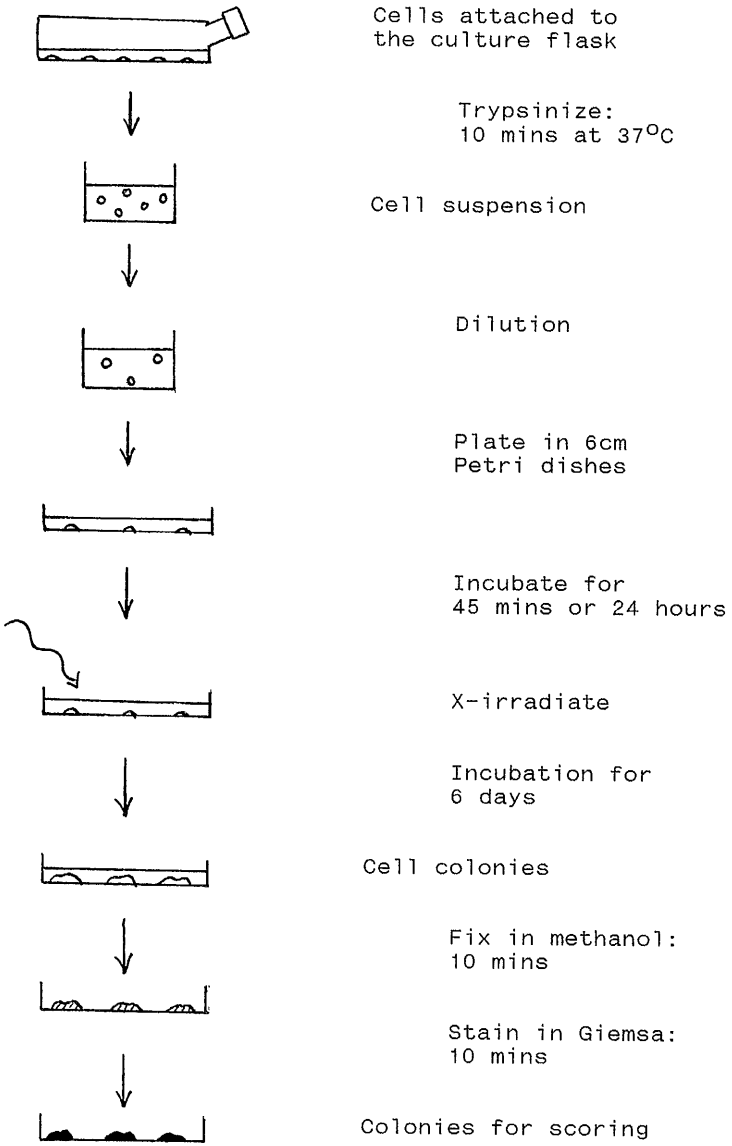


Figure 3.1 Clonogenic assay method for CHO cells - attached culture

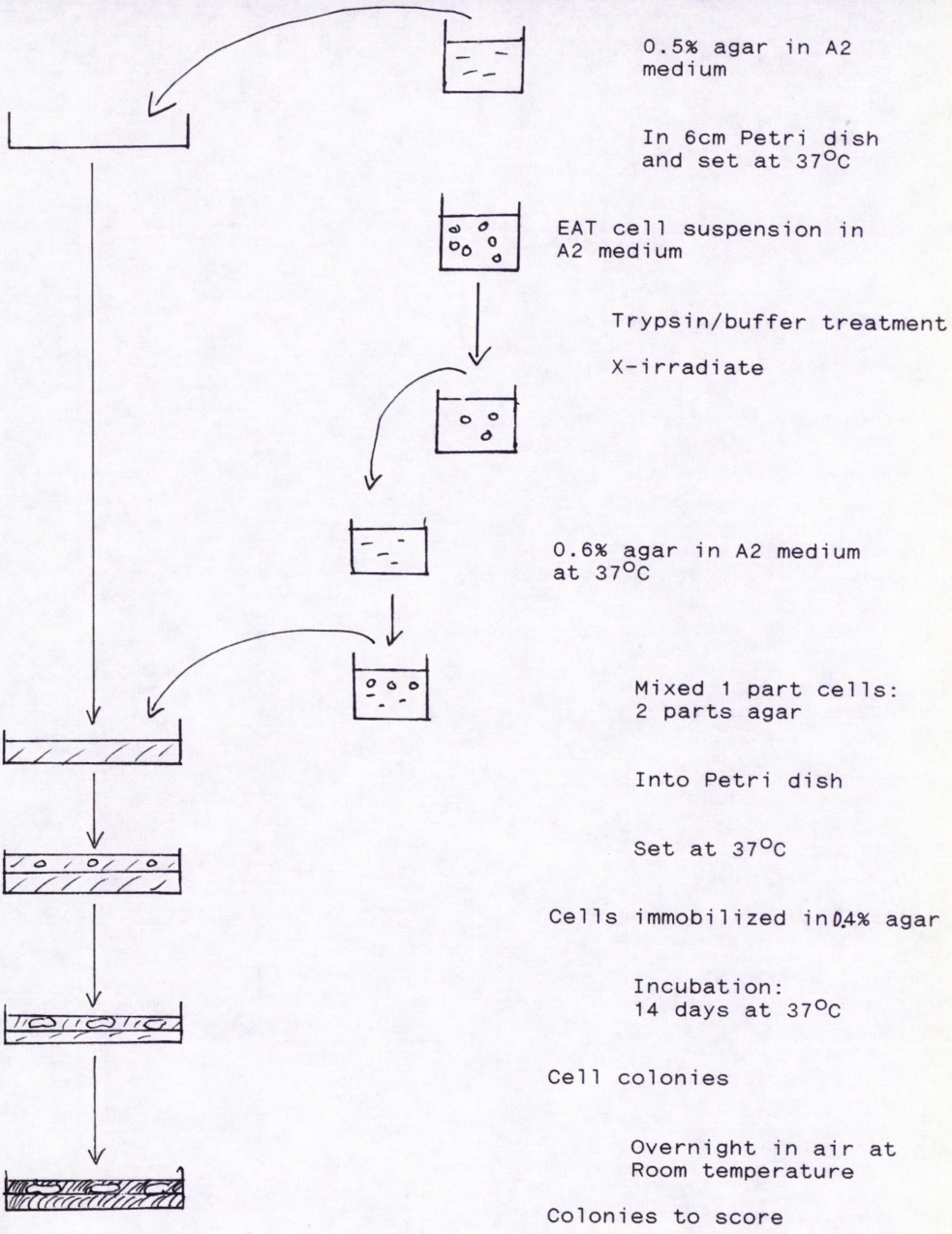


Figure 3.2 Clonogenic assay method for EAT cells - suspension culture

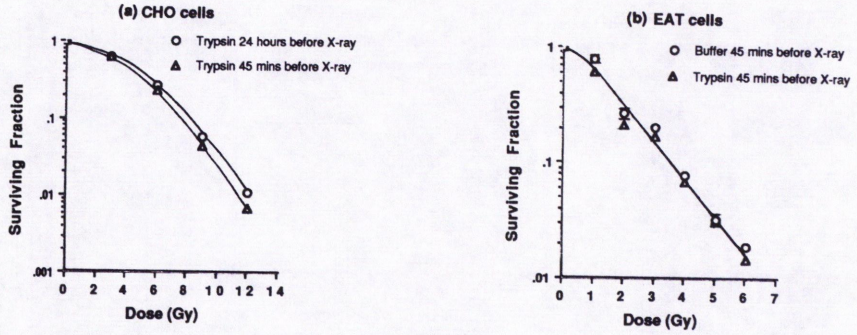


Figure 3.3 Survival curves. Bars show standard error of mean; where not shown are within symbol size.
The results of 2 experiments are shown in each curve

best-fit curve was drawn between both sets of points (D_o=1.5 Gy, D_q=0.6 Gy). The plating efficiencies for CHO cells were 62% when trypsinizing 45 minutes before irradiation and 98% when trypsinizing 24 hours before irradiation. For EAT cells, the plating efficiencies were 68% for those trypsin treated and 69% for those buffer treated. When studying the surviving fraction leaving CHO cells for up to one hour between

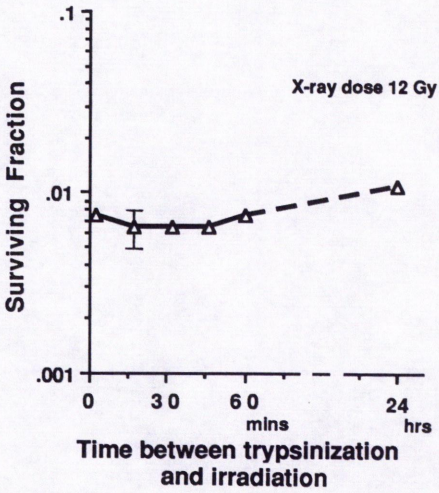


Figure 3.4 Change in CHO cell survival due to X-irradiation at different times after trypsinization. Bars show standard error of mean; where not shown are within symbol size.
The results of 2 experiments are shown

trypsinization and irradiation, a slight increase then decrease in sensitivity was seen (Figure 3.4) but was barely significant.

4. Discussion

Trypsin does potentiate X-ray cell killing in CHO cells, confirming earlier reports with other cell lines (Elkind *et al*, 1961; Lehnert, 1975; *etc*).

When trypsinizing after irradiation, this loss of potentiation of cell killing with time was interpreted as the fixation of PLD to LD by trypsin (Sun *et al*, 1986), the reduction in potentiation as the time between trypsinization and irradiation is increased being due to partial recovery from PLD before the damage is fixed. Possibly an analogous effect occurs when cells are trypsinized before irradiation, trypsin inducing changes in the cellular structure from which the cell may recover if enough time can elapse between trypsinization and irradiation.

The difference in trypsin effect between CHO cells as shown here and V79 cells (Sprunt, 1986) is significant and would not have been expected as both cell lines are derived from Chinese hamster fibroblasts and their radiosensitivities are similar. Berry *et al* (1966) saw a larger potentiation of cell killing in CHO cells than was seen here, but no multiplicity corrections were made in the data so it is not possible to compare their results with the results shown here. These results show that all attached fibroblastic cells do not demonstrate the trypsin effect to a similar extent.

The presence of the cyclic change in X-ray sensitivity to trypsin as the time interval between trypsinization and irradiation was increased was postulated to be due to differing sensitivities of different phases of the cell cycle to irradiation (Berry *et al*, 1966). No such dramatic effect was seen here for CHO cells as has already been shown for CHO cells over longer time intervals (Berry *et al*, 1966), partly because the cells

would not have gone back into cycle within one hour of trypsinization and also because the results of Berry *et al* (1966) again did not include a multiplicity correction so the true effect would be smaller than as they demonstrated.

The plating efficiencies in the CHO experiments showed that the post-plating treatment of unirradiated cells can affect cell killing, possibly also as a consequence of the cells having been recently trypsinized. Almost all cells survived if they were incubated at 37°C soon after trypsinization and plating and not disturbed until 24 hours later when all dishes were removed from the incubator whether due to be irradiated or not. The plating efficiency of those cells removed from the incubator 45 minutes after trypsinization, however, dropped to less than two thirds of the above value. No such effect was seen for EAT cells which were trypsin or buffer treated at the same time before irradiation.

EAT cells have not previously been used to study the trypsin effect; no potentiation was seen in this line which adds weight to the theory of Reddy *et al* (1989) that the change in morphology after cells are trypsinized corresponds to their change in radiosensitivity as no morphological change will be seen for EAT cells. As EAT cells grow in suspension, this result implies that the trypsin effect on cell killing at least is a phenomena of attached cells, adding weight to the argument that the trypsin effect is due to the trauma of cells being removed from the dishes, leaving part of their surfaces behind and the major morphological changes involved (Lamb and Ogden, 1987). The recovery from the trypsin effect could, as Elkind *et al* (1961) and Reddy *et al* (1989) suggested, coincide with the cells reattaching and flattening on the culture dish surface again.

The data does not refute the suggestion of Bender and Gooch (1961) who thought that irradiation of cells in suspension (*ie* after

trypsinization of attached cells) could damage the cell attachment mechanism, so even though the cells were viable they could not attach to form colonies so cell survival appeared to be lowered.

Chapter 4

INVESTIGATION OF THE TRYPSIN EFFECT BY ASSAYING FOR CHROMOSOMAL ABERRATIONS

1. Introduction
2. Materials and Methods
3. Results
4. Discussion

Chapter 4

1. Introduction

There is thought to be a relationship between chromosomal aberrations (CA) and cell killing (Bender *et al*, 1974) so an investigation into the effects of X-ray induced CA may provide information regarding the site of action of trypsin.

CA are thought to result from DNA double-strand breaks (dsb). Bender *et al* (1974) based their hypothesis on eukaryotic chromosomes containing one long double helix of DNA. Ds breakage of this was clearly not visible during interphase but at mitosis when the chromosomes condense the dsb could be seen as CA (Bender *et al*, 1974; Evans, 1977). These dsb may either not be repaired, resulting in deletions (breaks), achromatic lesions (gaps) and acentric fragments, or mis-repaired, yielding exchanges. These structural rearrangements have been discussed fully (Savage, 1976; Buckton and Evans, 1981). This is a more sophisticated version of the 'breakage-first' model devised by Sax (1940). An alternative hypothesis (Preston, 1980; 1982) is that DNA base damage, not dsb, results in CA. It cannot be completely ruled out that some CA result in this way, but this idea is not now in favour, and Obe *et al* (1982) have provided further evidence for the hypothesis of Sax (1940) and Bender *et al* (1974), showing that high LET (densely ionizing) radiation which is very efficient in DNA dsb induction is also very efficient in the induction of CA. Additionally, Bryant (1984; 1985) has shown that if restriction endonucleases (which are thought to cause DNA dsb) are introduced into cells, CA result.

Ionizing radiation is known as an S-independent agent as it can induce chromatid or chromosome damage; which of these is seen depends

on the stage in the cell cycle at which the damage occurred and the time after damage of sampling. If the cell is irradiated before S phase (*ie* in G_0 or G_1) then the damage will be copied exactly at replication resulting in both chromatids of the chromosome showing exactly the same damage and at metaphase will result in a chromosome aberration. If the cell is irradiated after S phase however, (*ie* in G_2) the DNA will have already been duplicated and so independent damage to each chromatid may be seen at metaphase. These are known as 'chromatid' aberrations. When using an asynchronous population therefore or cells in S-phase a mixture of chromosome and chromatid aberrations will be seen. Cytogenetic damage is thought to be lethal to the cell (*eg* Joshi *et al*, 1982).

Radiation damage can be mimicked by treatment of permeabilized cells with restriction endonucleases (RE) (Bryant, 1984; 1985) and techniques involving prior trypsinization of attached cells have been used to permeabilize cells to RE (Obe *et al*, 1985; Obe and Winkel, 1985), the effect of the RE then being investigated by assay for chromosomal aberrations (CA). Trypsinization is not essential to the permeabilization process as RE will also enter non-trypsinized suspension cultured lymphocytes (Obe *et al*, 1987) but trypsin treatment could result in more efficient entry of this enzyme into the cell as well as the RE. If trypsin entered the cell and acted on chromatin at routine trypsinization then more trypsin may enter the cell at permeabilization and its action would be amplified. If a trypsin effect was evident when assaying for CA then trypsin could therefore be potentiating the RE damage in these experiments but to a greater degree, an effect which has not yet been considered important in cytogenetics. It was decided therefore to investigate the effect of trypsinization on the incidence of X-ray induced CA to determine its significance without prior permeabilization.

As CA are thought to be connected with cell killing (Dewey *et al*, 1970; 1971; Carrano, 1973) the effect of trypsin on their induction could provide useful results in relation to the the clonogenic assay findings. In addition, the effect of trypsin on individual cells may be studied using this technique to determine whether trypsin influences all cells to the same extent or has a larger damaging effect on a selected population. Sun *et al* (1986) showed that the percentage of cells permeable to trypan blue (indicating membrane damage) after trypsinization was far higher for cells trypsinized and irradiated than the sum of the percentage of cells trypsinized or irradiated individually implying that trypsinization before irradiation increases the percentage of damaged cells. It will therefore be useful to investigate how the chromosomal effects on individual cells relate to this. Levan and Biesele (1958) observed an increased incidence of chromosomal abnormalities after trypsinization without irradiation, but they used a more aggressive trypsinization for their studies than is now used routinely in cell culture.

It was decided to use the EAT anaphase aberration assay (Bryant, 1983) as EAT cells grow in suspension, which means that meaningful non-trypsinized (buffer-treated) control samples could also be prepared for comparison. EAT cells are not suitable to assay for metaphase aberrations because the presence of many small chromosomes and their telomeric structure made scoring metaphases unreliable. A relatively high number of well defined anaphases are seen in a typical EAT cell population, the proportion of which is greatly increased if the cells are allowed to go into stationary phase (see chapter 2), inducing synchrony in G_0 phase, then incubated in fresh medium, harvesting during the wave of synchronized mitoses some 26 hours later. If the cells are irradiated before incubation in fresh medium, anaphase aberrations will

then be seen. CA are thought (Bender *et al*, 1974) to originate from DNA dsb which are not repaired (resulting in acentric fragments) or mis-repaired (resulting in exchange-type aberrations). Irradiation of cells in G_0 produces chromosome-type aberrations. A wide variety of aberration types are not seen at anaphase (Conger, 1965), just acentric fragments (unrepaired breaks) and bridges (dicentrics, or interchromosomal translocations). The formation of these is illustrated in Figure 4.2. Dicentrics result in bridges at anaphase as shown. A fragment is usually seen associated with a bridge, having been produced as part of bridge formation but can also be formed independently. Conger (1965) showed that approximately 50 per cent of asymmetrical exchanges result in an anaphase bridge and fragment, while fragments not associated with a bridge are as a result of chromosome deletions.

2. Materials and Methods

Anaphase abnormalities were assayed in EAT cells. The technique used was as described previously (Bryant 1983). A stationary population of EAT cells were prepared (see Chapter 2) and treated with trypsin or buffer for 10 minutes. Some cell samples were irradiated with an X-ray dose of 4 Gy, then all cell samples were resuspended in fresh medium, incubated at 37^{O}C and thus allowed to go back into cycle.

Cells were harvested between 21 and 28 hours from the start of incubation at one hour intervals to encompass the peak of the first mitosis after irradiation. The harvesting procedure was as follows. EAT cells were transferred to a 10 ml 'V' centrifuge tube and centrifuged for 5 minutes at 1000rpm in an MSE bench centrifuge, after which the supernatant was aspirated without disturbing the pellet. The pellet was gently resuspended, then 0.075 mol/l KCl ('hypotonic' solution) was added at room temperature for 10 minutes and the tube was centrifuged again.

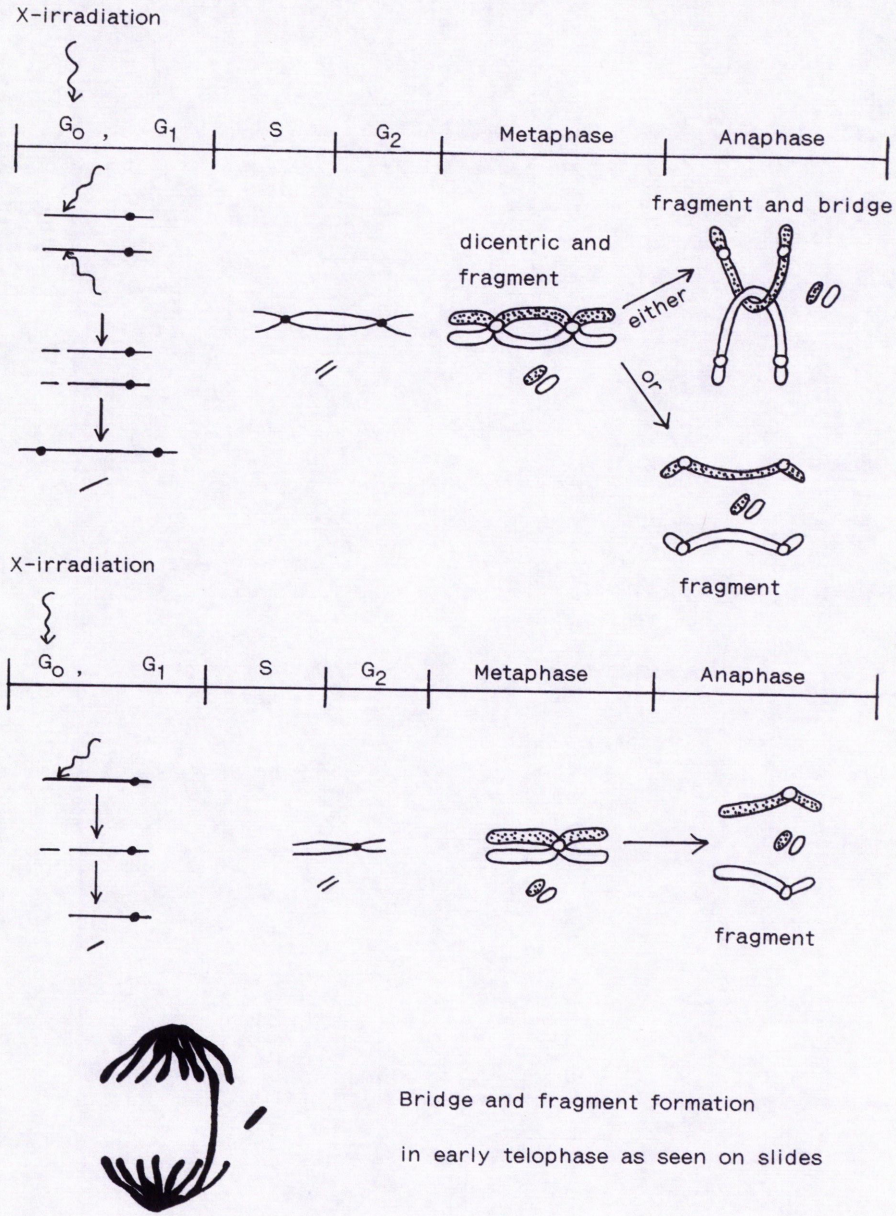
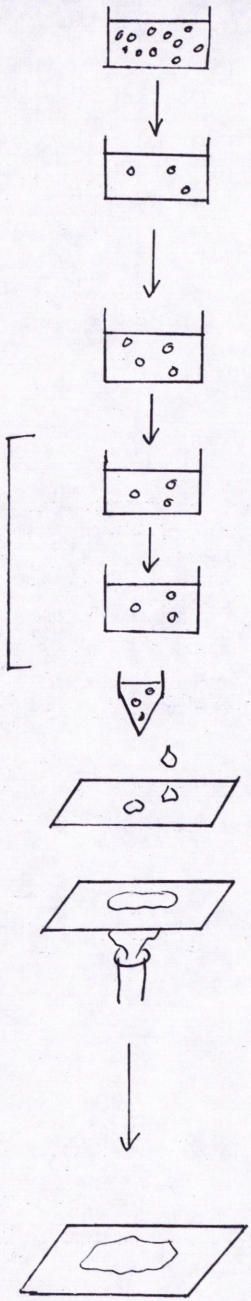


Figure 4.2 Anaphase bridge and fragment formation



Cells in stationary (G_0) phase

Trypsin/buffer treatment
X-irradiation
Resuspend in fresh medium

Incubate for 21-28 hours

Synchronized cells in mitosis

Spin out of medium; resuspend in 0.075M KCl - 'hypotonic solution'

10 mins at room temp.

Repeat 3 times

Resuspend in Carnoy's fixative
20 min at room temp.
Spin out of fixative

In fresh fixative overnight at 4°C
Spin out of fixative

Resuspend in 0.5ml fixative

2-3 drops on slide

Dry gently over flame

pH 6.4 buffer rinse
5 mins

3% Giemsa stain: 5 mins

pH 6.4 buffer rinse
1 min

Air dried at room temp

Figure 4.3 EAT anaphase aberration assay method

The KCl was aspirated and the cells were resuspended in 0.5ml fresh KCl. Carnoy's fixative (3 parts methanol to 1 part glacial acetic acid) was prepared freshly for each experiment. Cells were resuspended in 5ml fixative, left for 15-20 minutes at room temperature and then centrifuged for 3 changes of fixative. The first 2ml was added dropwise each time while agitating the tube. Cells were then stored overnight in fixative at 4°C. To spread the cells on slides, cells were centrifuged, the fixative aspirated and resuspended in approximately 0.5ml fresh fixative. 2 or 3 drops of the cell suspension were dropped onto each slide at room temperature then the slide gently dried over a flame. A high degree of spreading as is necessary for metaphase preparations is not required as individual chromatids do not need to be distinguished and a higher cell density allows for more efficient scoring. Once dried, the slides were rinsed in buffer at pH 6.4 for 5 minutes then stained in 3% Giemsa in pH 6.4 buffer for 10 minutes, rinsed in buffer and air dried at room temperature.

400 anaphases were scored per sample for abnormalities at one hour intervals over mitosis, scoring being carried out using a 40x objective on a Zeiss microscope. Very early anaphases were not scored as the chromosomes had often not separated enough for detail to be seen of the bridges; telophases were scored if they were still decondensed enough for the aberrations to be visible. Cell cycle phases in late mitosis, and a typical bridge and fragment as seen on the slides are shown in Figure 4.1. 1000 mitoses were scored per sample to determine the mitotic index.

3. Results

Stationary cells were incubated in fresh medium after treatment and after some 15-18 hours they entered cycle, the subsequent wave of

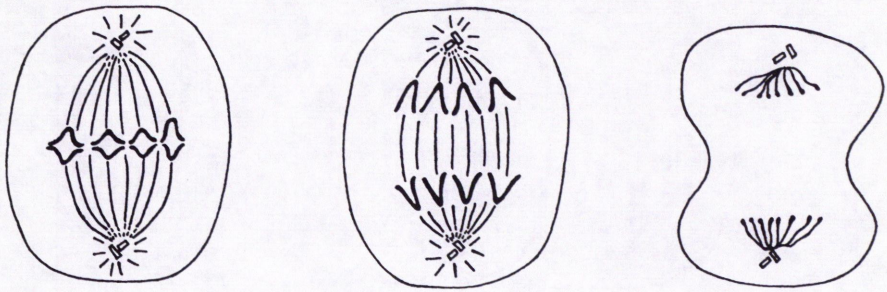


Figure 4.1 Cell cycle phases in late mitosis.

mitoses peaking at 24-26 hours (Figure 4.4). The percentage of normal anaphases increased slightly through the 21-28 hours incubation from 24.9% to 30.7%. When using unirradiated cells, 72% normal anaphases (0.4 aberrations per cell) were found in the trypsin treated samples and 69% normal anaphases (0.43 aberrations per cell) in those buffer treated. When similarly treated cells were X-irradiated with 4 Gy a difference was seen in the percentage of normal anaphases (Figure 4.5a) and in the average number of aberrations per cell (Figure 4.5b) between trypsin

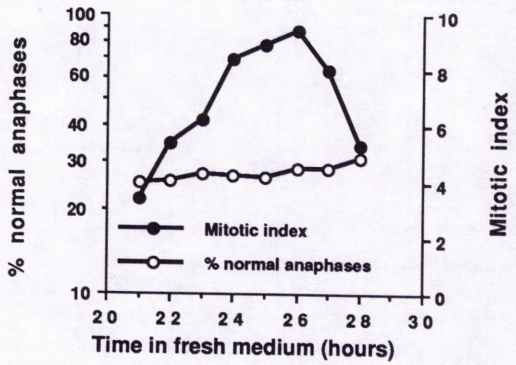


Figure 4.4 Percentage of normal anaphases and percentage of cells in mitosis both against time in fresh medium.
The results of 1 experiment are shown

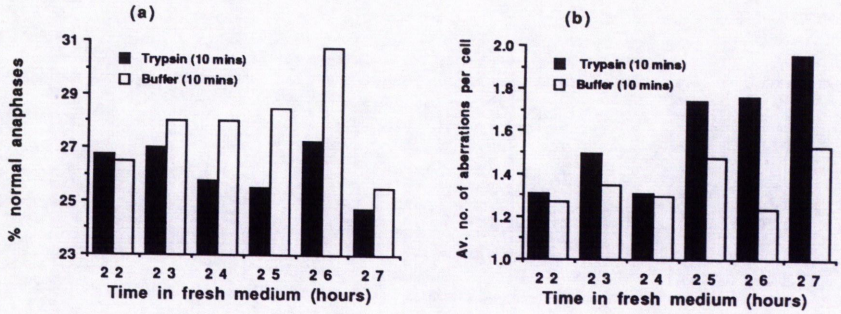


Figure 4.5 The effect of trypsin or buffer treatment on the incidence of anaphase aberrations after an X-ray dose of 4 Gy. (a) as percentage of normal anaphases and (b) as average number of aberrations per cell, both against time in fresh medium.

The results of 2 experiments are shown; one harvesting at 22, 24 and 26 hours and the other harvesting at 23, 25 and 27 hours (explained in text)

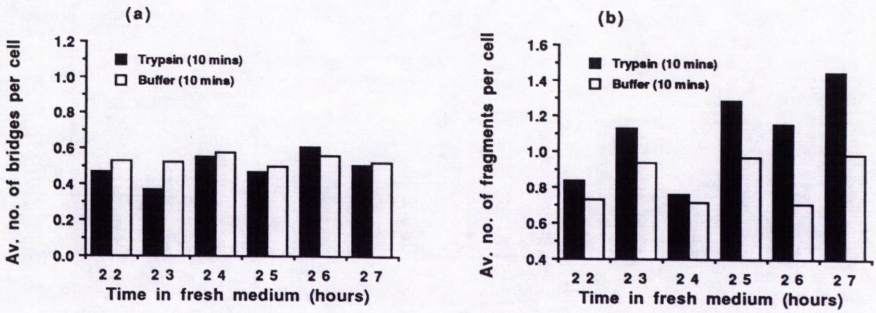


Figure 4.6 The effect of trypsin or buffer treatment on the incidence of (a) bridges and (b) fragments after an X-ray dose of 4 Gy. Both histograms are drawn to the same scale to aid comparison.

and buffer treated samples. Scoring of the irradiated samples was far less repeatable than in those unirradiated, the absolute number of aberrations varying considerably even though the increase in aberration frequency of trypsin treated cells over those buffer treated was repeatable, making it difficult to combine experimental data. The trypsin effect observed was generally consistent, but the background aberration level was variable. To illustrate this point, Figure 4.5 shows the results of 2 experiments for comparison: 22, 24 and 26 hours from one experiment and the odd hours from another. The only aberrations seen were acentric fragments (unrepaired breaks) and interchromosomal translocations (bridges); their incidences at each incubation time after 4 Gy is shown in Figure 4.6. The average number of bridges per cell was almost constant over the range of incubation times and between trypsin and buffer-treated samples and unexpectedly was marginally smaller in the trypsin treated samples. Trypsin did however induce a significant increase in the average number of fragments per cell over those buffer treated. From Figure 4.6a the experimental variability appears to be due to background levels of the average number of fragments per cell. The average number of aberrations per cell in the trypsin treated cells increased with time of incubation of the cells in fresh medium; this trend is not so pronounced in the buffer treated cells.

4. Discussion

Trypsin treatment was found to increase the number of anaphase aberrations in irradiated EAT cells over those EDTA/PBS treated. The increase in average number of anaphase aberrations per EAT cell with time of incubation in fresh medium could be due to the progressive accumulation of cells in anaphase with time; alternatively trypsin treatment could be altering the time at which the maximum aberration

frequency occurs, for example by inducing cell synchrony, the phases of the cell cycle having different X-ray sensitivities (Berry *et al*, 1966). It would have been useful to determine the aberration frequency over a wider range of incubation times to investigate these ideas but this was not possible as the mitotic index was too low at other incubation times for there to be enough anaphases to score.

The percentage of normal anaphases and the average number of aberrations per cell both show a trypsin effect of similar order of magnitude which implies that trypsin acts not only by increasing the damage to cells which are already abnormal but by increasing the frequency of abnormal cells in a population; Sun *et al* (1986) noted a potentiation of the frequency of membrane-damaged cells after trypsinization and irradiation compared with irradiation alone.

These experiments illustrate the importance of considering the trypsin effect in cytogenetic experiments as trypsin treatment may significantly affect the aberration frequency. If routine trypsinization potentiates CA incidence due to X-rays then an even more significant effect could be seen in RE experiments where cells are permeabilized just after trypsinization. Trypsin could potentiate this damage by opening up more restriction sites in the DNA; Tuschy and Obe (1988) saw a potentiation of RE-induced chromosome aberrations after hypertonic salt treatment of cells, which was thought to be due to salt causing a partial removal of histones from the DNA which opened up more RE recognition sites. Alternatively, a higher CA incidence could be seen if trypsin only acted on the cell membrane, as prior trypsinization could increase cell permeability to such an exent that significantly more RE could enter the cell to cause more chromosome damage. Further experiments assaying for CA after RE treatment of trypsinized and non-trypsinized suspension

cultures (such as lymphocytes) would be an important follow-up to this work.

Joshi *et al* (1982) concluded that the loss of reproductive integrity of cells was due to the loss of chromosome fragments after the first mitosis, while the presence of a chromosome bridge at the first anaphase after irradiation was thought by Carrano (1973) to be related to cell killing by preventing the separation of the daughter cells. Chromosome fragments can arise from misrepair *or* unrepaired breaks while anaphase bridges are solely derived from misrepair. The results here showed no increase in the incidence of chromosome bridges after trypsin treatment compared with those samples buffer treated after irradiation; in fact, there appeared to be marginally *fewer* bridges in the trypsin treated samples than in those buffer treated. Trypsin did however significantly increase the incidence of chromosome fragments. If trypsin potentiated cell kill by increasing the rate of mis-repair, an increased frequency of bridges should arise which was not seen. If trypsin inhibited repair however, less mis-repair would also occur and so a decreased frequency of bridges (and their associated fragments) would be expected, while an increase of independently-formed fragments would be seen. A marginal decrease in the incidence of bridges was seen after trypsin treatment, and an overall increase in fragment frequency. There does therefore seem to be more evidence for trypsin increasing the incidence of CA by inhibiting repair than by amplifying mis-repair. The clear increase in fragment frequency after trypsin treatment should reflect an increase in cell killing if the theory of Joshi *et al* (1982) holds. This will be discussed in relation to the cell survival experiments in Chapter 7.

Chapter 5

INVESTIGATION OF THE TRYPSIN EFFECT BY STUDYING THE INDUCTION AND REPAIR OF DNA SSB AND DSB

Chapter 5

1. Introduction

A trypsin effect was not detected in EAT cells using clonogenic assay while a significant effect was seen when assaying for anaphase abnormalities. To gain information on the reason for this discrepancy, the induction and repair of DNA strand breaks were investigated.

When X-rays pass through cells the resulting damage to cell components occurs at random, but DNA damage is thought to be the critical lesion which, if not repaired, will lead to cell death. The most common forms of X-ray damage are: single-strand breaks (ssb), double-strand breaks (dsb) and base damage (*eg* depurination or change in base structure). Other types of damage do occur but much less frequently, such as sugar damage, cross-linking, *etc.* The number of ssb and dsb induced are proportional to X-ray dose; in mammalian cells ssb are induced at a frequency of approximately 1120 ssb/Gy/genome, about 20 times more frequent than dsb which are induced at a frequency of approximately 40 dsb/Gy/genome (Freifelder, 1966; Corry and Cole, 1968; Dean *et al*, 1969; Lehmann and Ormerod, 1970; Burrel *et al*, 1971; Neary *et al*, 1972; Blocher, 1982). X-rays are a low LET radiation and only deposit relatively small amounts of energy in the form of electrons along their path, so they are much more likely to produce ssb than dsb in the first instance. Dsb are rarely induced in cells except after very high X-ray doses. Base damage occurs at around the same frequency as ssb.

In addition to the pure ssb and dsb, alkali labile sites must also be considered. When DNA is irradiated, some sites become damaged but without a ssb or dsb, such as apurinic/apyrimidinic sites (Collins, 1987) or sugar damage (Ahnstrom, 1988). If the DNA is then put in an alkaline environment, this converts the base damage to ssb, while in neutral

solution (pH 7-8), the lesion will remain just as base damage and will not be converted to ssb. Various techniques, *eg* neutral elution (Bradley and Kohn, 1979) are carried out at pH 9.6 to make the technique more sensitive as both base damage and DNA breaks are detected; a dsb could result if this site were near another ssb on the opposite DNA strand.

Many techniques are now well established in radiobiology to investigate DNA and chromatin damage and repair - see reviews by Kohn (1979), Ahnstrom (1988) and Radford (1988).

Repair mechanisms in mammalian cells are able to repair X-ray induced DNA damage. Ssb repair is very rapid ($t_{1/2}$ 5-30 mins *eg* Bryant and Blocher, 1980) and efficient, as there is usually a complementary sequence on the opposite DNA strand to act as a template (unless the opposite strand is base-damaged). The most common form of ssb is the 'dirty' ssb - that is, the break is surrounded by base damage, and is assumed to be repaired via the excision repair pathway as has been well characterized after UV damage. The damaged region is first excised, then new bases are inserted by DNA polymerase to replace the damage. The 2 ends are then joined up by DNA ligase to result in a fully repaired ds helix. Ssb are not thought to be causal in cell death. Base damage occurs at approximately the same frequency as ssb; as base damage is converted to ssb during the repair process it can be grouped with ssb when considering repair.

Kaufmann and Briley (1987) showed that the excision repair of trypsinized cells is 30% less efficient than for those in monolayers, and postulated that trypsin could alter the structure of the chromatin-DNA substrate and so affect the repair process. This effect was also observed by Collins (1987).

Dsb repair is thought to be more complex than ssb repair since both DNA strands are broken. Possibly as a result of this the repair of dsb is slower ($t_{1/2}$ of 2-4 hours, *eg* Blocher and Pohlit, 1982). Dsb

repair kinetics obtained using different techniques give conflicting results as is shown in Table 5.1. Using sedimentation and DNA unwinding the dsb were seen to be repaired at a constant rate (Lehman and Stevens, 1977; Bryant and Blocher, 1980; Blocher *et al*, 1983), whereas neutral elution yields biphasic repair kinetics (Woods, 1981; Weibezahn and Coquerelle, 1981; Sigdestad *et al*, 1987; Kemp *et al*, 1984; Radford, 1983). As a result of kinetics found in neutral elution experiments, it has been postulated that there may be more than one kind of dsb repair, *ie* those repaired by fast and slow mechanisms (Weibezahn and Coquerelle, 1981; Woods, 1981). The rapid component of dsb repair is postulated to be repaired by a simple process like blunt-end ligation, while the slow component may require a more complex repair process, possibly involving recombination (Resnick, 1976; Krasin and Hutchinson, 1977; Szostak, 1983). What is important about DNA dsb is that one unrepaired dsb is thought to lead to cell death (Blocher and Pohlit, 1982). Evidence that dsb are potentially lethal events is that high-LET radiations induce cell killing and DNA dsb with higher efficiency than low LET radiations (*eg* van der Schans *et al*, 1983) Also Bryant (1985) showed that RE (which only induce dsb) can cause cell death and chromosomal aberrations.

It was important to study dsb and their repair as this is thought to be the critical radiation-induced lesion involved in cell killing and so may be directly connected to the trypsin effect.

Neutral elution was used as this is a sensitive technique, dsb being detected in the survival curve dose range (Bradley and Kohn, 1979). Most techniques lyse and elute at pH 9.6 (Bradley and Kohn, 1979) but successful experiments have also been reported at pH 7.2 (Sigdestad *et al*, 1987; Evans *et al*, 1987) and pH 7.6 (Okayasu and Iliakis , 1989), albeit with altered kinetics. pH 9.6 is usually used as then, proteins which could be surrounding DNA breaks and so masking

the damage would be removed; this is not thought to occur so efficiently at a lower pH (Bradley and Kohn, 1979). However, at alkali-labile sites, base damage will be converted to DNA breaks at pH 9.6, so both base damage and dsb will be detected, not just pure dsb. Although this does mean that at this pH the technique will be more sensitive to DNA damage, pure dsb will not be measured (Evans *et al*, 1987). Alternatively, by lysing and eluting at pH 7.2-7.6, the alkali-labile sites will not be converted to DNA breaks; during cell lysis, proteinase k is added which should remove the proteins which would otherwise be removed at a higher pH and which could be masking the presence of dsb. Pure dsb are therefore thought to be detected at pH 7.2 (Evans *et al*, 1987). The interpretation of neutral elution has been discussed (Radford, 1988).

Reports of differences in dose-effect and repair kinetics between elution at pH 7.4 and 9.6 have been conflicting. Sigdestad *et al* (1987) reported that the elution dose-effect was slightly less sensitive at pH 7.2 than at pH 9.6, but the repair kinetics at pH 7.2 was exponential (linear on a log plot) with a half time of repair of approximately 102 minutes, although the 250 Gy X-ray dose used was far higher than X-ray doses typical of most radiobiological experiments. Okayasu and Iliakis (1989) did not find a difference between the dose-effect curves at pH 7.6 and 9.6; additionally, Koval and Kazmar (1987) found little difference between the repair kinetics at pH 7.2 and 9.6.

An unrepaired dsb is thought to lead to cell death (Blocher and Pohlit, 1982) or a chromosomal aberration (Bryant, 1985), so the detection of pure dsb by eluting at pH 7.4 may provide useful information regarding the trypsin effect.

The DNA unwinding technique (Ahnstrom and Erixon, 1973) is another sensitive method which detects the total strand breakage (ssb and dsb) including alkali labile sites. At low doses ssb can be assumed

to be the only lesion present, while dsb repair may be studied by irradiating the samples with a larger X-ray dose and allowing ssb to be repaired before sampling. The unwinding kinetics may also be used to study changes in DNA conformation. Even though ssb are not thought to correlate with cell killing their study may provide additional information on the mode of action of trypsin.

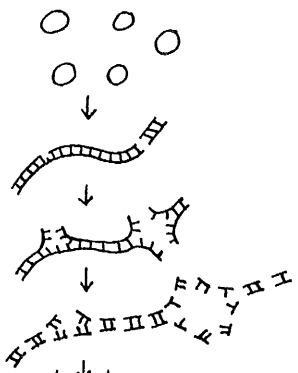
2. Materials and Methods

2.1 The DNA unwinding assay

The DNA unwinding assay (Ahnstrom and Erixon, 1973; Ahnstrom and Edvardsson, 1974) was performed essentially as described by Bryant and Blocher (1980). EAT cells at a concentration of $3x10^5$ cells/ml were labelled with $2x10^3$ Bq/ml ^{3}H-thymidine (specific activity $1.5x10^{12}$ Bq/mmol); 5μMol/ml unlabelled thymidine was also added. Enough cells were labelled to allow for at least $2x10^6$ cells per sample in the experiment. Cells were then incubated for 40 hours to allow labelling. Cell suspensions were treated with trypsin or buffer as described in Chapter 2 and kept on ice from this stage onwards except during repair experiments. It was important to pipette the cells well after trypsin treatment, especially after longer incubation times as the cells tended to stick in a pellet which, if not separated, would reduce the mean rate of unwinding. Cells were then irradiated with a graded series of doses to give a dose effect curve.

For repair experiments, cell samples were X-irradiated with 12 Gy or 50 Gy and incubated at 37^{o}C for time intervals between 0 and 60 minutes in bijou bottles in a water bath (short term repair) or 2-8 hours in non-tissue culture petri dishes in a CO_2 incubator (long term repair); the air supply in a bijou bottle is not sufficient to support efficient repair for several hours. DNA unwinding detects both dsb and ssb, data obtained being proportional to the total number of breaks in

DNA UNWINDING



^{3}H-LABELLED CELLS

IRRADIATE

LYSIS IN 0.03M NaOH;
1M NaCl

ULTRASOUND

ON HYDROXYLAPATITE
COLUMN

0.125M
PHOSPHATE
BUFFER

ss DNA
elutes

THEN: 0.25M
PHOSPHATE
BUFFER

ds DNA
elutes

COUNT BOTH FRACTIONS IN THE SCINTILLATION COUNTER TO DETERMINE THE AMOUNT OF SS AND DS DNA.

Mds/MtotDNA IS INVERSELY PROPORTIONAL TO THE NUMBER OF DNA BREAKS PRESENT IN THE DNA BEFORE UNWINDING.

Figure 5.1 DNA unwinding method

the DNA. Ssb induction and repair is investigated by using low doses so few dsb will be induced; to investigate dsb repair, samples are irradiated with a large (*eg* 50 Gy) X-ray dose, then left for 2 hours to repair, by this time ssb repair should be virtually complete so just dsb repair may be studied and the remaining damage should be within the sensitive detection range of the technique.

Cells were then pelleted (5 minutes at 1800rpm and $4^{o}C$ in a Fisons 'Chilspin 2'), the medium aspirated and the cell pellet resuspended. 5 ml ice-cold 0.15 M saline was added to each sample and the tubes were centrifuged again, the saline aspirated and the pellet resuspended. This centrifugation ensured that no medium remained; the medium has a buffering effect and phosphates which would neutralise the weak alkali, so the expected DNA unwinding would not be observed.

1 ml of 0.03 M NaOH in 1M NaCl (pH 12.5) was added forcefully to each sample which were then left in the dark for 1 hour without moving to allow unwinding as DNA in alkali is extremely sensitive to mechanical shock and UV light. The alkali must be prepared freshly for each experiment as the solution gradually absorbs CO_2 from the air - the alkali solution is so weak that this rapidly alters the pH of the solution and so affects the unwinding rate. X-irradiation will have induced breaks into the DNA. The unwinding solution causes the cells to lyse, then the high pH induces the 2 DNA strands to unwind at the break points. If there are many breaks in the DNA there will be many points at which unwinding occurs and so a high proportion of the DNA will be ss.

To investigate the unwinding kinetics all cell samples were X-irradiated with 12 Gy then the length of time for which the DNA was 'unwound' with alkali varied from 1 to 60 minutes.

The samples were each neutralized with 2 ml 0.02 Mol/l NaH_2PO_4 then sonicated in a 'Soniprep 150' MSE sonifier for 6 seconds with the

power amplitude set to 10μ. This breaks the DNA into small pieces at random-themorebreaks originally in the DNA, the higher the proportion of ss DNA pieces and the lower the proportion of ds DNA pieces in the sample. 0.03 ml of 2.5% SDS solution was then added forcefully to each sample to help maintain DNA separation and to remove any associated proteins, then all samples were frozen at -20°C. This freezing step is not essential but if the samples are not to be completely processed in one day in every experimental run, all samples should be frozen for consistency.

A 50:50 mix of DNA grade (very fine) and HTP grade (coarser; binds DNA less well) hydroxylapatite was prepared. This mixture gave a good flow through the column and good DNA binding for the number of cells used.

The various molarity phosphate buffers used to elute ss and ds DNA from the column were all diluted down from a 0.5M stock solution. This was prepared by making up 0.5M solutions of NaH_2PO_4 and Na_2HPO_4 and mixing them until a solution of pH 6.8 was obtained.

A 2 ml syringe body was used for the chromatography column, with a porous plug at the bottom to stop the gel passing through and a needle at the end for the DNA to drip through. The end of a Pasteur pipette held approximately 0.15g of hydroxylapatite which was put in each column. The columns were then put in place in holes in an aluminium block heated to 60°C.

The hydroxylapatite crystals were rehydrated with 2.5 ml low molarity (0.0125 Mol/l) phosphate buffer, and were stirred with a blocked Pasteur pipette to help produce a uniform column and remove bubbles. The solution was then allowed to drip through under gravity.

The frozen samples were quickly thawed at 37°C and poured onto the columns. The solvent dripped through, leaving all the DNA (ss and ds) bound to the column, thought to be by interaction with constituent

Ca^{++} ions. The column was once again washed with 0.0125 Mol/l phosphate buffer.

Ss DNA was eluted from the columns into scintillation vials with two washes of 2.5 ml 0.125M phosphate buffer. Then fresh scintillation vials were put under the columns and ds DNA was eluted with two washes of 2.5 ml 0.25 Mol/l phosphate buffer.

The DNA had been initially labelled with tritium, so the mass of DNA in each sample was determined by detecting the amount of radioactive label in each vial.

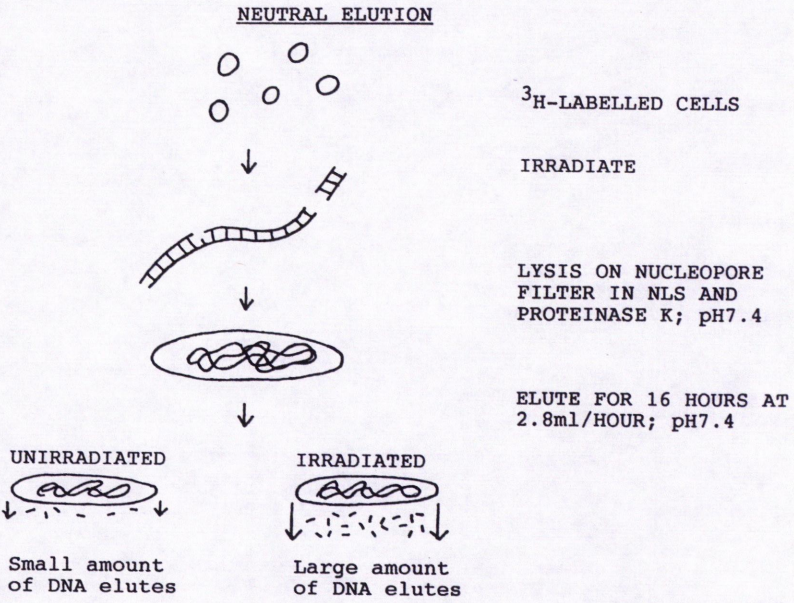
0.3 ml of 5 Mol/l HCl was added to each vial - this helps the phosphate buffer to absorb the scintillant. Then 5 ml Packard 'insta-gel' scintillant cocktail was added. This contains a strong detergent to keep the DNA separated.

The amount of radioactivity (*ie* DNA) in each vial was determined by counting the samples in a 1219 Rackbeta 'Spectral' liquid scintillation counter. The counter had an automatic quench correction, so the results were given directly as disintegrations per minute, directly proportional to the mass of DNA in each vial.

By determining the mass of ss and ds DNA resulting from each dose, the proportion of the total mass of DNA that is still double stranded ($mass_{ds}/(mass_{ds}+mass_{ss})$DNA) may be determined - this is inversely proportional to the total number of breaks (ds and ss) present in the DNA before unwinding.

2.2 Neutral Filter Elution

The non-unwinding DNA filter elution technique was used as developed by Bradley and Kohn (1979) with minor modifications (Okayasu and Iliakis, 1989) and is thought to detect only dsb. Exponentially growing EAT cells were diluted to $3x10^5$/ml and labelled for 40 hours with $4x10^3$ Bq/ml ^3H (specific activity $1.5x10^{12}$ Bq/mmol); 50μmol/ml



COUNT THE ELUTANT AND FILTER IN THE SCINTILLATION COUNTER TO DETERMINE THE FRACTION OF THE TOTAL DNA WHICH HAS ELUTED. THIS GIVES AN INDICATION OF THE NUMBER OF DSB IN THE SAMPLE.

Figure 5.2 Neutral elution method

unlabelled thymidine was also added. After cell treatment $5x10^5$ cells were pipetted into each 40 ml syringe body which was then connected to a 2 μm Nucleopore polycarbonate filter held in a Millipore swinnex holder. The medium was allowed to drip through the filter under gravity to load the cells onto the filter. When almost all the medium had dripped through, 10 ml PBS was then added to each syringe barrel and allowed to drip through completely. Lysis of the cells on the filter was then carried out for 1 hour at 60°C in 1 ml of 2g/l sodium-N-laurylsarcosine (NLS) and 0.5mg/ml proteinase K (Sigma) set to pH 7.4 or 9.5. This should ensure cell lysis, total separation of DNA from proteins and other structures and total loss of DNA tertiary structure. After lysis, the DNA was eluted from the filters with 40 ml tetrapropylammonium hydroxide set to pH 7.4 or 9.5 at approximately 2.8 ml/hour (15-16 hours' lysis). Where there were many dsb in the DNA the strands were fragmented into many small pieces and so were easily drawn through the filter. When the DNA was not damaged however, the strands were very long and would not pass through the holes, therefore, the fraction of the total DNA present which had eluted as determined by radioactive labelling gave a measure of the number of dsb present. Fractions were collected for each 3 hour interval over this time. The amount of activity eluted in each vial was determined by counting in a scintillation counter after adding Optiphase MP scintillation cocktail (LKB) and the amount of activity remaining on each filter counted after the addition of 5 ml Filter Count (Packard) to each vial. The amount of activity eluted from each filter (proportional to the mass of DNA eluted) as a fraction of the total activity in each sample (filter activity plus eluted activity - proportional to the total mass of DNA) gives a measure of the DNA damage to each sample. The background fraction eluted (f_e), that is, the unirradiated value, was subtracted from each f_e value initially calculated to give the values quoted here.

3. Results

3.1 DNA Unwinding Kinetics

The DNA unwinding kinetics are shown in Figure 5.3. No difference was seen in the unwinding kinetics of trypsinized and buffer-treated cells after a 12 Gy X-ray dose.

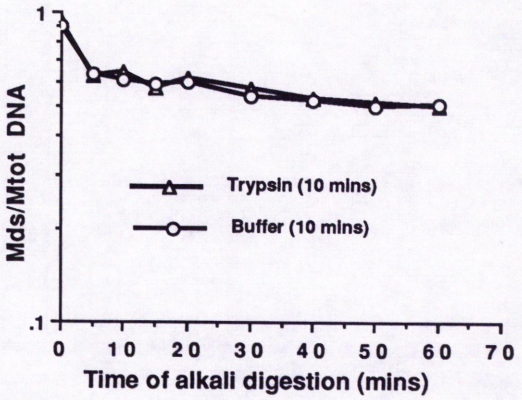


Figure 5.3 The effect of trypsin or buffer treatment on the DNA unwinding kinetics after an X-ray dose of 12 Gy.
The results of 2 experiments are shown

3.2 Dose Effect Curves

DNA unwinding dose-effect curves were compared for trypsin and buffer treated and untreated EAT cells under various conditions; the results of which are shown in Figure 5.4. No significant difference in the rate of induction of ssb with dose was seen between the treatments. Published data suggests a straight line relationship for the dose effect curve - my data also approximates to a straight line. Chasing the cells for 4 hours before commencing the experiment was not found to improve the dose-effect curve and so was not used (results not shown).

Neutral elution was first carried out at pH 7.4 and 9.5 to select which would be most suitable for this investigation. Dose effect curves

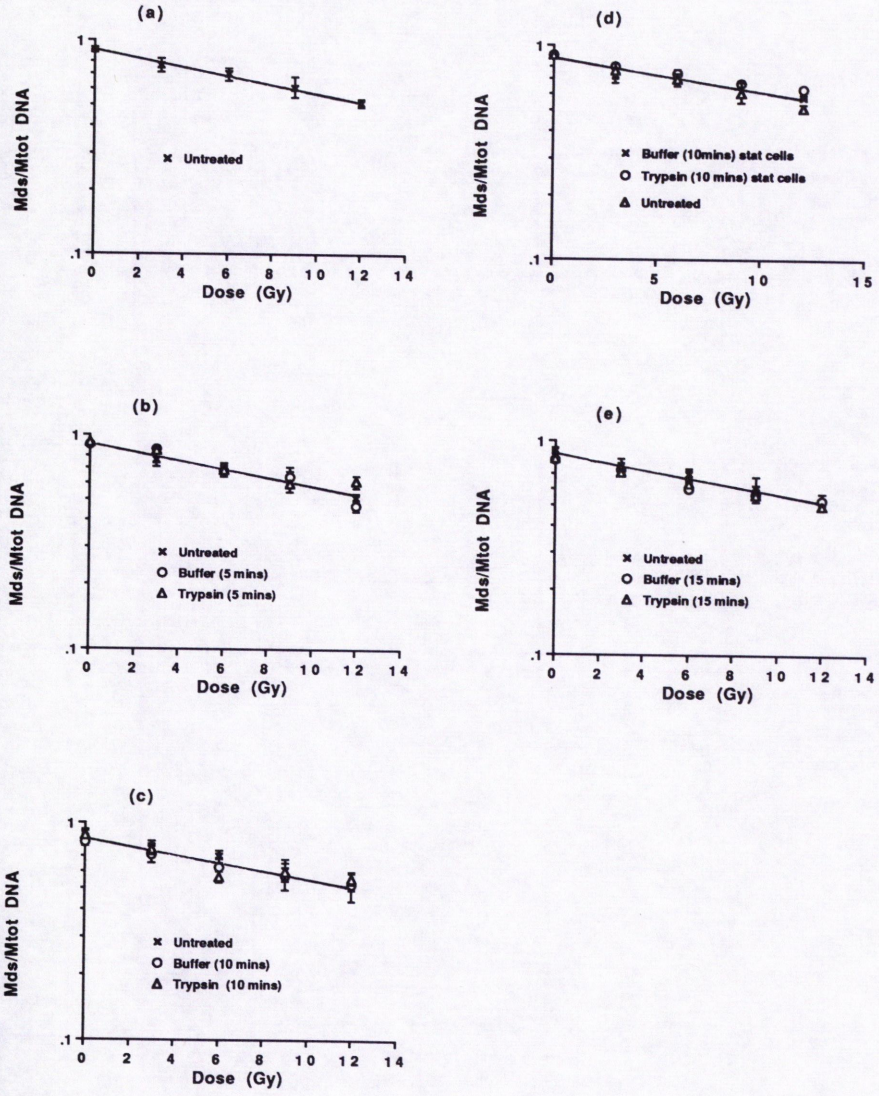


Figure 5.4 DNA unwinding dose-effect curves after various cell treatments.
The results of 2 to 4 experiments are shown in each set of data

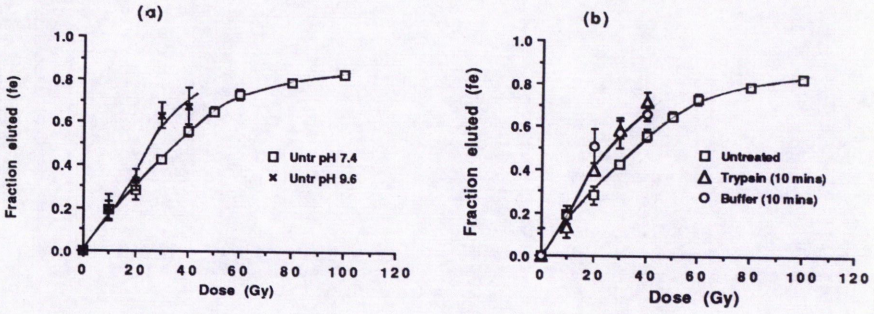


Figure 5.5 Neutral elution dose-effect curves.
The results of 2 to 3 experiments are shown in each set of data

carried out at pH 7.4 and 9.5 are shown in Figure 5.5a. Although the pH 9.5 curve gave a steeper response in the 30 - 40 Gy dose range and a larger difference in f_e overall so may be more sensitive, the pH 7.4 curve was approximately linear over the 0 - 40 Gy dose range to be studied here and would be more comparable with the nucleoid experiments also carried out at pH 7.4 (see Chapter 6), so this pH was used for the investigation. At both pHs the dose effect curves eventually flattened at higher doses, contrary to published results but in agreement with other workers in this group (Costa and Bryant, accepted subject to revision).

Using neutral elution the dose-effect curve for induction of dsb showed no difference in response between trypsin and buffer treated cells, but these both showed a steeper response than untreated cells (Figure 5.5b). This result was unexpected since neither trypsin nor buffer treatment altered the DNA unwinding dose-effect curve.

3.3 Short term repair kinetics using DNA unwinding

The ssb repair kinetics of EAT cells after X-irradiation with 12 Gy were obtained as M_{ds}/M_{tot} DNA (results not shown) and converted to remaining damage using the relevant dose effect curve to aid

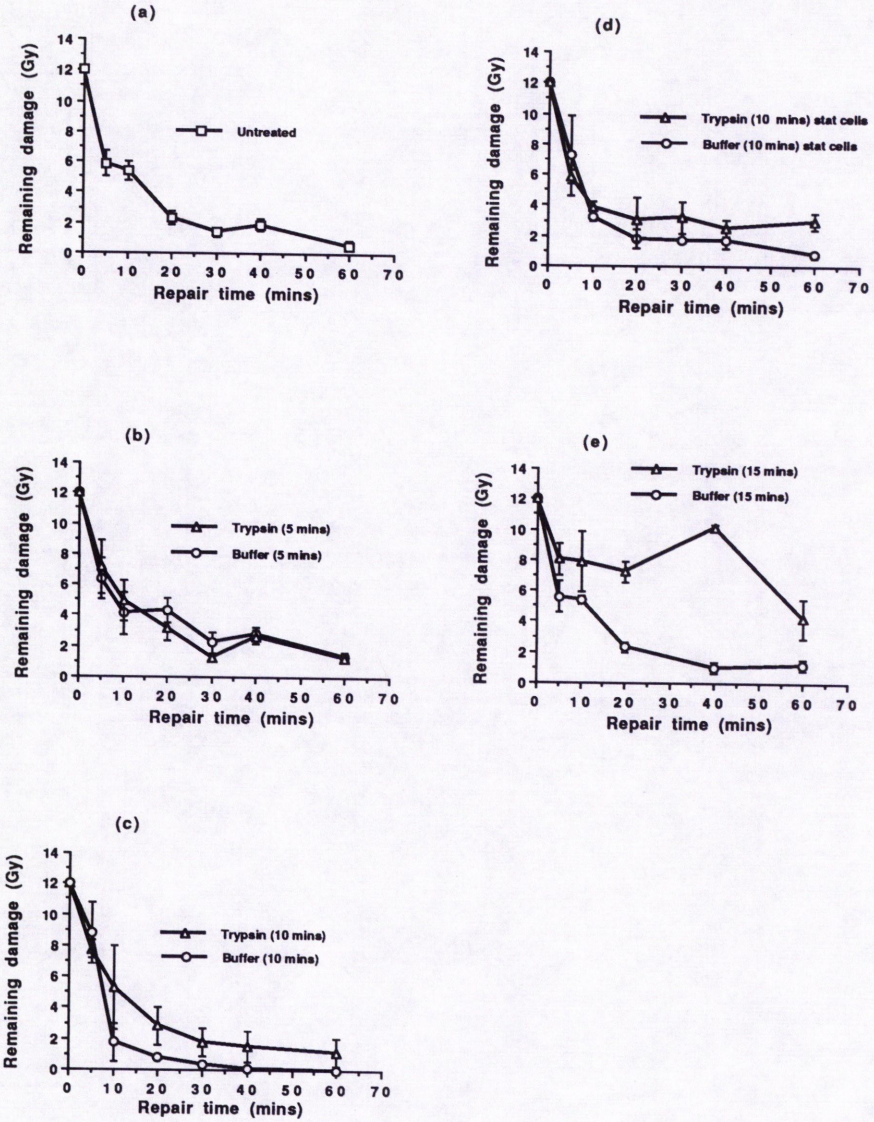


Figure 5.6 Short term (ssb) repair kinetics. The results of 2 to 3 experiments are shown in each curve

interpretation. The ssb repair kinetics are shown in Figure 5.6. There is clearly some variation in the background repair levels in experiments; it is the difference between the trypsin and buffer treated curves which must be primarily considered. No significant difference in the repair kinetics of untreated and 5 minute trypsin or buffer treated cells was seen; a small difference was seen after a 10 minute treatment and the repair of the 15 minute trypsin treated cells was much slower than those untreated. No evidence was found to suggest a difference in ssb repair in buffer treated and untreated cells. No significant difference was seen in the repair kinetics of exponential and stationary cells. Chasing was not found to improve the repair kinetics and increased the uncertainty in each point (results not shown) so was not pursued.

3.4 Long term repair kinetics

Using DNA unwinding, no difference was seen between the repair of trypsin and buffer treated samples, but these both repaired less efficiently than the untreated samples (Figure 5.7a).

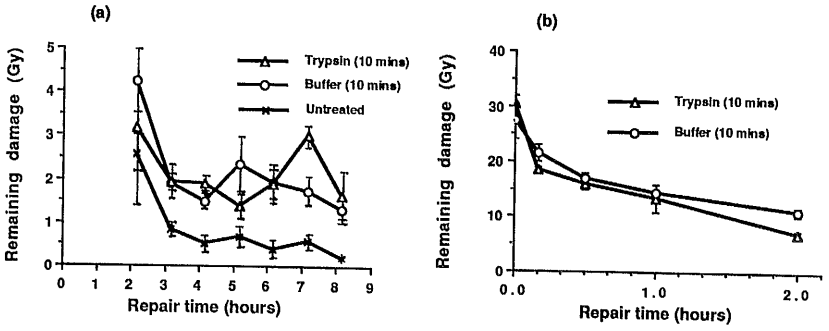


Figure 5.7 Long term (dsb) repair kinetics. The results of 2 to 3 experiments are shown in each curve

To further investigate whether there was a difference in dsb repair between trypsin and buffer treated cells, the dsb repair kinetics were checked using neutral elution. A 40 Gy X-ray dose was used as

this was the highest dose for which the dose response was still linear. The repair kinetics may be studied from t=0 using neutral elution as dsb are the only form of damage detected.

The repair kinetics (Figure 5.7b) confirmed those obtained with the DNA unwinding method in that the kinetics after 40 Gy showed no significant difference between the repair of trypsin and buffer treated cells.

Even though a 40 Gy X-ray dose was given, the remaining damage at t=0 was only 30 Gy. This could be because the 'no repair' cells were left on ice for the 2 hours while the repair experiment was carried out then irradiated at the end, which could have affected the cells adversely, but this would be more likely to damage the cells, thus rendering them less capable of repair than the reverse. Published neutral elution data generally does not include a non-repair sample, the repair kinetics originating from the point representing the known dose given (Evans, *et al*, 1987; Sigdestad *et al*, 1987).

The half times of repair comparing these results and other published results are shown in Table 5.1.

4. Discussion

No difference was seen between the DNA unwinding kinetics of irradiated trypsin and buffer treated and untreated cells, implying that there is no major conformational change in the chromatin after trypsinization with or without irradiation to visibly alter the rate of unwinding of the DNA at the break points. The theory of trypsin interacting with the histones and causing the DNA to expand or the theory of Reddy *et al* (1989) that chromatin contraction is involved with the trypsin effect cannot cause a significant enough effect to show up here.

Table 5.1. Half times of repair comparing thesis and published results.

Technique and Treatment	Half Time Initial	Half Time Final	Source
DNA unwinding ssb repair			
Untreated	5 mins	24 mins	Thesis
Untreated	5 mins	30 mins	Bryant and Blocher, 1980
15 mins trypsin	7.5 mins	1 hour	Thesis
DNA unwinding dsb repair			
All	45 mins	2.5 hours	Thesis
	2-4 hours		Bryant and Blocher, 1980
Neutral elution dsb repair			
pH7.4 all	15 mins	1 hr 50 mins	Thesis
pH9.6	5-10 mins	0.5-2 hours	Weibezahn and Coquerelle, 1981
			Woods et al, 1982
			Blocher et al, 1983
pH7.2	102 mins		Sigdestad et al, 1987 (250 Gy dose)
	15 mins	160 mins	Sweigert et al, 1989

Trypsin or buffer treatment did not increase the rate of ssb induction with X-ray dose above the untreated levels, but the neutral elution results implied an increase in dsb induction at higher doses in trypsin and buffer (EDTA) treated cells compared with those untreated. Possibly trypsin and EDTA potentiate the induction of dsb but not ssb. Another possibility is that trypsin and EDTA affect the ability of damaged DNA to elute through the filters (*eg* by a decrease in viscosity) rather than increasing actual DNA damage. A variation in the neutral elution dose response at various phases of the cell cycle has been demonstrated (Okayasu *et al*, 1989) which was thought to be due to alterations in DNA conformation affecting the elution. The effect was mainly thought to be due to DNA replication, although small differences were also seen in the dose response curves of G_1 and G_2 cells. No significant difference was seen between the induction of dsb in trypsin and buffer (EDTA) treated cells. Either trypsin has no major potentiating effect here and it is the EDTA which causes the potentiation of induction of dsb (or change in viscosity), or the potentiation effect is saturable so trypsin does not induce any additional damage.

When investigating the repair of ssb using DNA unwinding, buffer (EDTA) alone had no effect on repair, whereas trypsin was found to progressively reduce the capacity for repair of ssb when increasingly longer incubation times were used. Kaufmann and Briley (1987) observed that the excision repair of trypsinized cells was 30 per cent less efficient than for attached cells, a similar effect also having been reported by Collins, (1987); the decrease in repair capacity seen here could be reflecting their observations. If an increase in cell permeability was inducing the decrease in ssb repair capacity, possibly after 10 minutes the trypsin has still only just begun to act on the surface proteins while after 15 minutes could cause more significant damage. These ssb effects should not however affect cell survival.

Reddy *et al* (1989) suggested that trypsinization could be causing chromosome contractions as seen in hypertonic saline-treated cells causing a fixation of X-ray damage and so an increase in cell kill. Bryant (1982) additionally showed that hypertonic treatment inhibited dsb repair using the DNA unwinding technique, concluding that inhibition arose from structural alterations in the chromatin. The dsb inhibition by both trypsin and buffer (EDTA), seen here compared with untreated samples in DNA unwinding, did not correspond with a change in unwinding kinetics which should demonstrate conformational alterations, so if chromosome alterations do occur they must be too subtle to be detected in this way. EDTA alone also inhibited dsb repair to the same extent so this must also affect chromatin. This concept has not previously been mentioned in the literature. DNA unwinding dsb repair kinetics generally show a constant half time of repair of 2-4 hours but the initial rate of repair was faster than this (45 minutes), see Table 5.1. This may be due to ssb still being repaired here, and it is not until after a repair time of 3hours that almost all the breaks left are dsb.

No separation in the trypsin and buffer treated dsb repair response was seen when using neutral elution, confirming the DNA unwinding results. Current theory suggests that there may be more than one kind of dsb repair - fast repair which acts by a simple blunt-end ligation and a slow component which is repaired by recombination. This is thought to be reflected in the two-component repair kinetics curve shown here. These elution results confirm reports of biphasic neutral elution repair kinetics at pH 7.4 (Koval and Kazmar, 1987; Swiegert *et al*, 1989) and do not support the observation of Sigdestad *et al* (1987) of exponential repair kinetics. The initial half time of repair was rather slower than that published for repair kinetics at pH 9.6 (see Table 5.1). This could partly be due to the fact that a no-repair sample

was included, unlike in most reported kinetics, which was found to give a value for remaining damage (Gy) significantly lower than the known X-ray dose given. If the X-ray dose given had been plotted rather than the experimental value, the initial half time of repair would have been significantly faster.

The observation that trypsinization causes no difference in the *induction* of breaks as determined by unwinding but does induce cells to have a lower capacity for *repair* of X-ray induced ssb implies that trypsin increases cell kill and the number of chromosomal aberrations not by potentiating the X-ray damage itself but by reducing the repair capacity of the cells after the damage is caused.

It appears that EDTA plays a major part in the potentiation as well as trypsin; in both dsb induction and repair, no additional effect was seen when using trypsin over that seen when using EDTA. It is possible that EDTA and trypsin affect dsb induction and repair, but just trypsin affects ssb induction and repair. Alternatively, the damage done by trypsin and/or EDTA could be saturable.

Chapter 6

INVESTIGATION OF THE TRYPSIN EFFECT USING A NUCLEOID ASSAY

4. Investigation into nucleoid morphology
   4.1 Dark field microscopy
   4.2 Fluorescence microscopy - staining nucleoids with ethidium bromide
   4.3 Bright field microscopy - staining nucleoids with Giemsa
   4.4 Bright field microscopy - staining nucleoids with 'Stainsall'
   4.5 Scanning electron microscopy
   4.6 Transmission electron microscopy
   4.7 Bright field and scanning electron microscopy - staining nucleoids with DNase
   4.8 Flourescence microscopy - immunoassay to locate the nuclear lamina
   4.9 Fluorescence microscopy - effect of varying the ethidium bromide concentration on the dimensions of triton DW nucleoids
   4.10 Cell diameter measurement before lysis

5. Comparison of the response of different lysis techniques to X-irradiation by scoring nucleoid dimensions
   5.1 Preliminary nucleoid scoring
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7. Discussion

Chapter 6

1. Introduction

1.1 Isolation of nuclear matrix residues

Various nuclear residue preparations with differing degrees of similarity to the *in vivo* matrix have been used to study different aspects of nuclear structure, such as nuclear matrix (Berezney and Coffey, 1974; Agutter and Richardson, 1980), ghost (Keller and Riley, 1976; Agutter and Richardson, 1980) scaffold (Paulson and Laemmli, 1977) and cage (Cook and Brazell, 1978). These various types of residual skeletal substructure were isolated using hypertonic salt treatment as part of the preparation procedure but were all different from each other and from the *in vivo* structure. The 'nuclear matrix' is isolated by extraction of nuclei with high salt, nuclease digestion and non-ionic detergent (Berezney and Coffey, 1974; Comings and Okada, 1976; Herman *et al*, 1978; Long *et al*, 1979; van Eekelen and van Venrooij, 1981). This structure consists of residues of the nuclear lamina, internal matrix and nucleolar structure but should not be confused with the *in vivo* nuclear matrix structure. Paine *et al* (1983) showed that when isolating animal cell nuclei in 'physiological' media, 50% of the nuclear protein was lost in the first 3 seconds which may well produce major changes in the nuclear structure. Nuclear isolation is also known to damage the DNA, so techniques used to study the organization of chromosomes by producing nuclear substructures involving initial nuclear isolation (*eg* Adolph, 1980) may not be illustrative of structures found in the living cell. Additionally, the removal of DNA as part of matrix preparation procedures is thought to induce artefactual structural changes to the remaining nuclear residue (Gallinaro *et al*, 1983). It is therefore

important to try to avoid these damaging stages to gain a more reliable impression of the interaction of chromatin with nuclear structures.

1.2 Current theories on nucleoid structure

The problem of the nuclei isolation stage was overcome by Cook and Brazell (1975) who developed a technique, similar to an independently devised method involving lysis of prokaryotic bacterial spheroblasts to isolate nuclear body derivatives (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972). Cells were lysed directly (non-ionic detergent, EDTA and 1-2M NaCl) on a sucrose gradient, which avoids the damaging nuclei preparation stage which is the major cause of controversy in nuclear matrix preparation; the resulting nuclear residues, termed nucleoids, were then isolated by centrifugation through the sucrose gradient. Most of the cellular material (*ie* protein) was removed but all the nuclear DNA and most of the nuclear RNA was still present; the resulting structures still resembled nuclei but they consisted of a fibrous protein derivative of the nuclear matrix known as the nuclear cage (Cook and Brazell, 1978) with intact DNA associated, and also some derivatives of the cytoplasm which protect the cage structure within as well as cytoplasmic RNA (Cervera *et al*, 1981; van Venrooij *et al*, 1981). Some of the proteins resemble those found in other nuclear preparations such as nuclear pore complex, lamina, envelope or matrix (Berezney and Coffey, 1974; Riley *et al*, 1975; Aaronson and Blobell, 1975; Comings and Okada, 1976) but the amount of DNA-associated proteins depends on the salt concentration used for lysis; if 2M NaCl is used no histones will remain. Nucleoids also contain cytoskeletal elements such as actin, keratin and intermediate filaments which have condensed onto the central structure (Levin, 1978). Nucleoids can be prepared from a wide variety of cell types from mammalian and bird cells to amphibian and insect cells (Cook and Brazell, 1976a), and similar techniques for

nucleoid production have since been used by a number of authors (*eg* Vogelstein *et al*,1980; Buongiorno-Nardelli *et al*, 1982). Methods have also been developed where nuclei are prepared prior to nucleoid production (Bekers *et al*, 1986; Buongiorno-Nardelli *et al*, 1982) which then does not make use of the main advantage of nucleoid production that nuclei do not have to be initially prepared.

'Nuclear cage' is accepted to refer to the non-chromatin residual structure of nucleoids produced by lysing cells in non-ionic detergent, EDTA and high (1-2M) NaCl concentration. Three types of attachment of DNA to the cage have been reported. The first is structural, maintaining the DNA in loops and exists throughout the cell cycle (Warren and Cook, 1978; Cook and Brazell, 1980; McCready *et al*, 1982). The other two are involved with replication (McCready *et al*, 1980; Vogelstein *et al*, 1980; McCready *et al*, 1982; Jackson *et al*, 1984) and transcription (Jackson *et al*, 1981; McCready *et al*, 1982; Jackson *et al*, 1984), these nuclear processes having been shown to occur at the nuclear cage.

Some authors also term cells lysed in SDS (an ionic detergent) and EDTA, 'nucleoids', with nuclear cages (*eg* Ostling and Johanson, 1987), but only 12% of RNA and less than 5% protein remained (Cook, 1984; Ostling and Johansson, 1984), so the same non-chromatin structure cannot be present. Lysing with ionic detergents results in nuclear residues with far larger halos in solution than triton-lysed nucleoids as virtually all the proteins from the DNA are removed so the DNA decondenses into long strands which may be easily damaged. If the cells are set in agarose before SDS-lysis, most cellular material other than the DNA will diffuse out of the hole into the agar. The DNA is then thought to expand to fill the hole left by the cell in the agarose but will not be able to diffuse out into the agarose unless there are strand breaks in the DNA (Ostling and Johanson, 1984); the extent to

which the DNA may be drawn out of the hole into the agar by electrophoresis therefore reflects the degree of breakage of the DNA (Ostling and Johanson, 1984). Various models for the structure of the central of SDS-lysed nucleoids have been put forward. Ostling and Johanson (1984) initially postulated that the central region was the boundary of the hole in the agar, with the DNA contained inside until strand breaks occur. Only after X-irradiation when the DNA is nicked can the damaged DNA then be drawn out of the centre by electrophoresis, the nucleoid centre being the undamaged DNA remaining in the agar hole, the degree of distortion of the staining material being used to obtain a dose response curve. No mention of a nuclear matrix was made. This theory was however later modified (Ostling and Johnson, 1987) to suggest that the central structure *was* a residue of the nuclear matrix and DNA exists in clusters of replicons including intact supercoiling after lysis. The technique was thought by the authors to show similarities to nucleoid sedimentation by the authors, and applying an electric field was thought to stretch out the relaxed loops in irradiated cells. This technique has been successfully used to assay for radiation damage by other authors (Olive *et al*, 1986; Olive, 1989). The connection with nucleoid sedimentation was taken a step further by Erzgraber and Lapidius (1988) who lysed cells with SDS on a sucrose gradient and isolated the resulting structures by sedimentation in 1M NaCl. Again it was assumed by the authors that the resulting DNA-protein complexes had supercoiled DNA Domains.

1.3 Nucleoids and DNA supercoiling

The DNA part of HeLa cell nucleoids remains tightly supercoiled within the nuclear cage and so protected and resistant to shear (Cook *et al*, 1976). If such cells with strong nuclear cages are irradiated to introduce nicks into the DNA then lysed in solution, or if unirradiated

cells with weak nuclear cages are lysed, this causes the DNA to be released from within the nuclear cage and spread out in a halo around the cage. A halo is also seen when nucleoids are spread prior to scanning electron microscopy. It has been shown that the eukaryotic genome in interphase nuclei (the 30nm fibre) is organized into numerous compact supercoiled loops, topologically anchored at their bases to the central cage (Cook and Brazell, 1975, 1976; Cook *et al*, 1976; Ide *et al*, 1975; Benyajati and Worcel, 1976; Vogelstein *et al*, 1980; Adolph, 1980; Lebkowski and Laemmli, 1982a and b, Igo-Kemenes and Zachau, 1978). The loop structure is studied by lysing cells in 2M NaCl which removes the histones, but the integrity of the DNA structure must still be maintained as the loops are still supercoiled and nucleohistone complexes can then be reconstructed on the nucleoid DNA (Levin and Cook, 1981). An analogous framework structure known as the scaffold is thought to form the 'backbone' of chromosomes at mitosis (Paulson and Laemmli, 1977; Adolph, 1977a; 1977b). The intercalation of increasing concentrations of ethidium bromide into the loops has been shown to cause the negative DNA supercoiling to be gradually lost (thus expanding the halo), then positive supercoiling to be introduced (compacting the loops again), causing the nucleoids to sediment in a biphasic manner (Cook and Brazell, 1975; 1976), which would not be possible if the loops were not topologically constrained, the loops binding ethidium like superhelical DNA (Cook and Brazell, 1978). It has been estimated that there is on average one supercoil every 90 to 180 base pairs (Cook and Brazell, 1977) in looped domains of approximately 220 kb in HeLa cells (Cook and Brazell, 1975; 1978) and 85 kb in Drosophila cultured cells (Benyajati and Worcel, 1976), although this figure is stated with caution as Ganguly and Bagchi (1984) have shown an increase in average domain size in chicken erythrocyte chromatin after mild salt treatment.

When cells are irradiated with X-rays (Cook and Brazell, 1975; 1978) or UV (Cook and Brazell, 1976; Vogelstein *et al*, 1980) the nicks induced into the supercoiled DNA loops allow them to unwind and so the supercoiling is lost. It is this loss of supercoiling which results in the loops spreading outside the nuclear cage making the structure less dense and so sediment a shorter distance down the sucrose gradient, the greater the X-ray dose, the shorter the distance sedimented. This is the basis of the nucleoid sedimentation technique first described by Cook and Brazell (1975) and since used by many authors (*eg* Van Rensberg *et al*, 1985; 1987; Schwarz *et al*, 1984).

Electron microscopic findings produced additional evidence of DNA supercoils (McCready *et al*, 1979). SEM and TEM studies of HeLa nucleoids spread using Kleinschmidt's (1969) technique (McCready *et al*, 1979) have been published. The cage collapsed, spilling the DNA loops out in a skirt surrounding the centre. All closed circular DNA molecules *in vivo* exhibit negative supercoiling (van Holde, 1989; supercoiling reviewed by Bauer *et al*, 1988); the DNA in these structures is slightly underwound and supercoiling is induced in eukaryotes by wrapping the DNA around nucleosome cores. The amount of proteins and histones associated with the DNA and so the conformation of the loops depends on the salt concentration of the lysis solution; HeLa nucleoids have been prepared at salt concentrations ranging from 0.4 to 2 M NaCl (Levin *et al*, 1978).

As for nuclear matrix isolation, the use of extreme non-physiological conditions, especially high salt, to produce nucleoids raised the argument that nuclear cage structures observed had been produced artifactually and do not relate to the *in vivo* system. In an attempt to overcome this, Jackson and Cook (1985a; 1985b) have also studied nucleoids prepared in isotonic salt and found that transcription (Jackson and Cook, 1985b) and replication (Jackson and Cook, 1986a; 1986b) still

occurred at the nucleoskeleton. The resulting residue was termed the nucleoskeleton as distinct from the nuclear cage when the structure was isolated in 1M NaCl. Low salt nucleoid production is however restricted to nuclear structure studies and is not used to produce nucleoids to study DNA damage and repair. Under these low salt conditions, Mirkovitch *et al* (1984) did not find that the transcribed regions of active genes were attached to the skeleton as is shown in high salt conditions (Ciejek *et al*, 1983; Cook *et al*, 1982; Robinson *et al*, 1982) but rather the *upstream* regions, the discrepancy being thought to be due to the sliding of the DNA relative to the scaffold at high salt concentrations, throwing some doubt on the above results using high salt. These upstream attachment regions have been shown to lie close to Topoisomerase II sites (Gasser and Laemmli, 1986).

1.4 Aim of this chapter

Most techniques used to detect the damage and repair of DNA after X-irradiation are based simply on the detection of DNA (ss or ds) breaks, detecting the mean effect on many cells. The true situation is, however, far more complex than this, since the DNA is not isolated within the cell but is part of a DNA-protein superstructure in close association with the nuclear matrix. Simply studying DNA breaks is therefore falling well short of the true complexity of the situation. When DNA is damaged it is likely to also affect those other structures with which it is associated and conversely, damage to surrounding structures may influence the damage to DNA. Additionally, after a break has been repaired the chromatin must return to its original conformation. Such conformational changes in DNA will also not be detected when simply assaying for strand breaks. Detecting conformational changes using the nucleoid sedimentation technique is sometimes considered to be a

disadvantage where simply the number of ssb is required (Boerrigter, 1989) but here we are also interested in conformational effects.

Loss of the potentiating effect of trypsin was not observed in selected mitotic cells when left for some hours before irradiation or cells treated with hypotonic salt (HS) solution immediately after irradiation (Reddy *et al*, 1989). It was postulated that trypsin may induce alterations to chromatin structure in exponentially growing cells similar to those induced by HS and mitosis - both of which have contracted chromosomes (Dettor *et al*, 1972). Tuschy and Obe (1988) observed a potentiation of restriction endonuclease-induced chromosomal aberrations by high salt concentrations which they suggested was due to a partial dehistonization of the chromatin, thus increasing enzyme recognition sites. Szekely *et al* (1983) showed that anisotonic treatment caused a conformational change in cellular chromatin, which could be related to the observed fixation of PLD in exponentially growing cells by anisotonic solution (Raaphorst and Azzam, 1981). DNA accessibility is known to influence the rate and extent of repair (Wheeler and Wierowski, 1983) so possibly the contraction of DNA is involved with the trypsin effect. Evidence against this is that trypsin treatment did not alter the DNA unwinding kinetics which is thought to highlight differences in DNA conformation. Studies of nucleoid structure after trypsinization may provide more information on the effect of trypsin on chromatin conformation. Variations on the amounts of ethidium bromide (EB) bound to different human carcinoma cell lines has recently been interpreted as illustrating varying accessibility of DNA to EB or alterations in DNA supercoiling within the nucleoid (Schwartz and Vaughan, 1989) and variation in the maximum size of a DNA loop was used to show that heat shock alters the DNA-nuclear matrix anchor points (Kampinga *et al*, 1989). It is possible that a similar conformational effect might be seen after trypsin treatment.

Trypsin is known to reduce the rate of UV-induced excision repair (Kaufmann and Briley, 1987; Collins, 1987); additionally, UV-induced lesions have been shown to be repaired at the nuclear cage (McCready and Cook, 1984) so trypsin could be acting at this level.

It was decided to further the investigation of the trypsin effect by developing a nucleoid direct observation assay to detect DNA damage in individual cells. Cook and Brazell (1975) were the first to describe the nucleoid sedimentation technique involving the gentle lysis of living eukaryotic cells in non-ionic detergent, tris-HCl, EDTA and NaCl to produce 'nucleoids' which were subsequently centrifuged in sucrose gradients to discrete bands. This nucleoid sedimentation technique was later modified for direct observation (Vogelstein *et al,* 1980; Roti Roti and Wright, 1987). The advantage of a direct observation technique is that the way in which a damaging agent is interacting with individual cells may be studied so it was decided to use such a technique rather than the original nucleoid sedimentation method. It may thus be determined whether such an agent (like trypsin) greatly increases the damage to a small proportion of the cells in a population, or whether it affects all cells to a lesser extent. Other advantages of the direct observation methods are that radioactive labelling (which could be interacting with the DNA) is not necessary, and few cells are needed as the effect of the treatment on every cell can be recorded. Also, unlike the chromosomal aberration assays, it is possible to look at the effect of X-irradiation without allowing repair to occur, or by allowing limited repair. This technique may thus give more information than simply the number of induced ssb, and may provide information on the effect of trypsin on the conformation of chromatin as well as the damage caused.

2. Factors affecting lysis and chromatin configuration

The aim of this study was to develop a new nucleoid assay based on those already published, especially if potential for automation was found, to determine more about the trypsin effect. The main factors to consider when devising this technique will be discussed in this section, including the reasoning behind its development. Nucleoid techniques generally use a solution of triton (0.5%), NaCl (1-2 M for dose-effect curves and repair; down to 0.15 M for structural observation) to lyse and solubilize the cell and nuclear membranes, EDTA to prevent cell nucleases digesting the DNA during lysis, and often tris-HCl as a buffer. This technique was based on such a lysis method, but other details were quite different.

2.1 Detergents used in nucleoid production

Before a detergent can act on the cell interior it must first cause cell lysis. Cell lysis occurs before membrane solubilization at a lower detergent concentration resulting from interaction between the detergent and the membrane lipids specifically, causing localized membrane disordering and is indicated by proteins and other macromolecules beginning to cross the membrane. Once the cell membrane has been lysed, detergents are then free to work on the cell interior, acting on the nuclear membrane in the same way.

The type of detergent will then greatly influence the way in which it interacts with proteins within the nucleus, including those associated with chromatin. Detergents possess a hydrophilic ('water-loving') head region (which may be ionic ,*eg* SDS, or non-ionic but polar, *eg* Triton X-100) and a hydrophobic ('water-hating') tail region. Ionic detergents are also known as denaturing detergents. These have a charged head and flexible apolar tail and so can interact with both the hydrophilic and

hydrophobic regions of molecules. Non-ionic detergents are also known as mild detergents (*eg* Triton X-100, one of the mildest), and interact predominantly by hydrophobic means.

When SDS binds to protein, this results in a drastic conformational change of the protein as all tertiary and quaternary structure is destroyed and previously buried hydrophobic groups are exposed as SDS disrupts virtually all non-covalent protein-protein, protein-lipid and DNA-protein (Noll and Stutz, 1968) interactions and so multi-sub unit proteins dissociate into their constituent polypeptide chains. The SDS-protein complexes form extended rod-like particles, the proteins being therefore rendered inactive, nuclease activity being reduced (Marmur, 1961). Ionic detergents such as SDS can lyse both cells and nuclei (Cook and Brazell, 1975) and have been shown to remove supercoiling from prokaryotic chromosomes (Worcel and Burgi, 1972).

Mild detergents are non-ionic and so only interact with hydrophobic regions of proteins. They also tend to have rigid and bulky apolar regions which probably cannot penetrate the crevices of the protein surfaces as efficiently as the flexible alkyl chains of denaturing detergents and so are very inefficient at breaking protein-protein interactions. This means that triton X-100 can solubilize most proteins without denaturing proteins into their sub units or inducing conformational changes even at high detergent concentrations, and many maintain their biological activity due to the detergent mimicking the lipid environment in the hydrophobic regions. Triton will not remove the histones from chromatin even when in excess; a high salt concentration must be added to the solution for this to occur. Non-ionic detergents can easily lyse cells but will not lyse nuclei (Cook and Brazell, 1975).

It was decided to use 2 extremes of detergent: triton X-100 (very mild, non-ionic) and SDS (very strong, ionic), for comparison, and also to try sodium lauryl sulphate (NLS), another ionic detergent found to be

very successful for lysis as part of the neutral elution technique, giving a lower background value than SDS; possibly nucleoid preparations may provide clues to why this is so. The advantage of using triton and SDS is that much work has been done on their action on the cell which aids the interpretation of results. Methods involving direct observation of nucleoids set in agar have also been successful in producing nucleoids by lysis with SDS (Ostling and Johanson, 1984) and triton (Roti Roti and Wright, 1987), obtaining dose-effect curves and repair kinetics.

Commercial detergents such as the triton used in this investigation are chemically impure, batches varying greatly, so all the triton used in this investigation was from the same batch. If detergents are left standing, the composition of the liquid will vary from top to bottom of the bottle so it must be well shaken before use.

2.2 Ionic strength of lysis solution

Varying ionic strength (generally discussed in terms of salt concentration) can have a dramatic effect on intracellular structures including chromatin. Changing the salt concentration will alter the number of double helical turns and so change the number of superhelical turns. At physiological pH, DNA has a net negative charge. At very low salt concentrations (<0.01 M) there are no ions present to shield the negatively charged phosphate groups on the DNA strands from each other, so the electrostatic repulsion will not allow the bases to approach each other, and only ss DNA is found. As the salt concentration increases towards physiological (0.15 M), the counter ions (Na^+) increase in concentration accordingly and cluster around the polyanion, shielding adjacent negatively charged phosphates in each ss of DNA so the 2 DNA strands can come together, the salt effectively stabilizing the H-bonds. When the DNA ss have come together to give ds DNA, positively charged histones (strongly attracted to the negatively charged DNA) can bind to

the ds DNA very tightly, to form the 10nm fibre at 0.15 M NaCl. Other DNA-associated proteins are also bound at this concentration. If the salt concentration is increased (up to 0.4 or 0.5M), chromatin can condense down still further to give the 30 nm fibre as intra-strand repulsion is reduced by the Na^+ ions continuing to shield the negatively charged phosphates. When the ionic strength exceeds 0.4M, the chromatin tertiary structure begins to break down as the ions begin to shield the histones and DNA from each other and so these electrostatic interactions break down. Firstly histone H1 is lost, reducing the chromatin structure back to the beads on a string structure, and above 0.5M, chromatin begins to dissociate into histones and DNA as the ions compete for the stabilizing hydrogen bonds and so break them. As the salt concentration is raised above 0.75M NaCl, first histones H2A and H2B are lost, followed by H3 and H4 (Levin *et al*, 1978; Levin and Cook, 1981). At 1M NaCl, variable amounts of histones are present, but by 1.95M, no histones and few other proteins characteristic of chromatin are still associated with the DNA (Cook and Brazell, 1976; Levin *et al*, 1978). High salt concentrations reduce the affinity of many other proteins for DNA and have been shown to remove loosely associated polypeptides from the nuclear matrix (Warters *et al*, 1986) and nuclear salt soluble proteins; some very tightly nuclear matrix associated proteins, however, stay associated at saturating concentrations of NaCl (Cook *et al*, 1976). Various salt concentrations have been used in triton lysis solutions to produce nucleoids, from 0.15M to 2M. Higher salt concentrations generally result in nucleoids with more fragile nuclear cages; 0M, 0.15M, 1M and 2M salt will be used here.

2.3 Lysis environment used in nucleoid production

Published nucleoid techniques using triton lysis to obtain an X-ray dose-response produce nucleoids by lysing cells in various environments, for example, on top of a sucrose gradient (nucleoid sedimentation - Cook and Brazell, 1975), lysing cells while growing attached to a microscope slide (Vogelstein *et al*, 1980) and lysing cells in wells pretreated with poly-l-lysine to aid attachment (Roti Roti and Wright, 1987). These preparations are all quite temporary, so only limited scoring is possible before the samples must be discarded. If cells could be successfully lysed with triton while set in agarose on a microscope slide and a method of obtaining a dose-effect devised, this could be preferable where direct scoring of nucleoid damage is to be carried out as it may be possible to maintain the samples for longer time intervals by sealing the slide to prevent dehydration, and such slides may be easier to handle than nucleoids in solution. The published SDS techniques set cells in LGT agarose made up in PBS (Ostling and Johanson,1984); nucleoids have also been successfully prepared by setting cells in agarose microbeads then lysing in triton, but this technique has not been used to obtain a dose-response.

It was decided here to attempt such a technique setting cells in agarose on a microscope slide before lysis, using PBS or distilled water (DW) to make up the agarose; DW would give the cells a hypotonic shock before lysis and may alter the morphology of the nucleoids.

2.4 pH of lysis solution

At great extremes of pH, the DNA double helix will undergo major disruption, but even over the limited pH range considered here, significant transitions will be seen in the chromatin structure.

The pH of Ostling and Johanson's (SDS) lysis solution was pH 9.5 - presumably to make the technique more sensitive by converting alkali-

labile sites to ssb. However, neutral elution was shown here to be successful at pH 7.4 as well as at pH 9.6 in this thesis, and it is argued that pH 7.4 will give a response more in keeping with the *in vivo* situation than will pH 9.6; all nucleoid sedimentation (triton-lysed) experiments are carried out at either pH 7.0 or 8.0. To gain information from these experiments on what is actually happening in the cell it would seem more useful to work at physiological pH, so it was decided to use pH 7.4 (physiological) for triton and SDS nucleoid preparations but also to use pH 9.5 for SDS nucleoid production to determine if there was any significant variation in morphology between the pHs.

2.5 EDTA concentration in lysis solution

EDTA is essential to all lysis solutions as it chelates Mg^{++} and Ca^{++} ions and so prevents them from acting as cofactors for nucleases which would otherwise be degrading the nucleic acids in the cell while lysis was progressing. It is desirable to use a high EDTA concentration to ensure that all Mg^{++} and Ca++ are chelated; the maximum EDTA concentration reported to be successful was 133mM (Cook, 1984), so this was also used in this investigation. Sufficient EDTA was especially important here as the lysis time required was very long (see Section 3.1.3); even a small nuclease activity could have a significant degradative effect over longer time intervals.

2.6 Buffered lysis solutions

A buffer system is often also added to the lysis solution. All the triton lysis solutions tend to include tris-HCl (*eg* Cook and Brazell, 1975) whereas those using SDS do not, so this trend will be maintained here.

2.7 Cell lines used for nucleoid production

Most of these techniques have used HeLa cells, an aneuploid cell line originating from a human cervical carcinoma. Their nuclear cages are very robust and the DNA will not spill out when in solution except after irradiation. These aqueous techniques are not, however, convenient for, *eg* primary diploid cells which have much more fragile nuclear cages which break easily, thus releasing the DNA which is then sheared (Cook and Brazell, 1976b). It was decided to attempt to devise a nucleoid technique which had potential for a wider range of cell lines, including those with weak cages; as the cells were to be set in agar this may afford some protection to the DNA. CHO cells were used here as this is a widely used cell line in radiobiology, so results from a nucleoid technique using CHO cells could therefore be compared with other results in other experimental systems.

2.8 Staining nucleoids

All published nucleoid sedimentation and direct observation techniques use a fluorescent stain to visualize nucleoids (*eg* Cook and Brazell, 1975; 1978; Vogelstein *et al*, 1980; Roti Roti and Wright, 1987). Although nucleoids stained in this way produce a reproducible dose-response by direct observation, scoring would be more difficult and take longer than when using a light stain, and the fading of the stain means that samples cannot be stored for any length of time before scoring; Roti-Roti (1987) lysed cells in triton and fluorescent stain, then photographed nucleoid preparations before scoring as the fluorescence would not last more than 24 hours. Flourescent stains are also inconvenient as the resulting 'glare' of the image reduces the visible detail and so makes scoring inaccurate and unreliable. The quantimet computer-linked microscope cannot be used with fluorescence, so it

would be an advantage to develop a staining method to use with the bright field microscope.

Vogelstein *et al* (1980) found that nucleoids would take up Giemsa stain successfully, but the nucleoid structure distorted during drying. The drying technique was not expanded upon, but the nucleoids were dried in solution not in agar which may have caused the problems; Ostling and Johanson (1984) found no distortion of their SDS-lysed nucleoids after drying in agar. Colman and Cook (1977) also stained nucleoids with Giemsa.

The main components of Giemsa are methylene blue and eosin. The active forms of methylene blue are basic dyes which impart a violet-blue colour to the acid components of the cell such as nucleic acids and nucleoproteins; eosin is acidic and so reacts with the basic components of the cell including several cytoplasmic constituents and haemoglobin, staining them red.

3. Materials and methods

3.1 Nucleoid preparation - general method

The technique is summarized in Figure 6.1.

3.1.1 Slide preparation

Microscope slides (Objettrager 'K' micro slides) were treated with 0.1% hot ultra pure electrophoresis grade agarose (BRL, no.5510UA) a few hours before the experiment was due to start and left to air dry at room temperature.

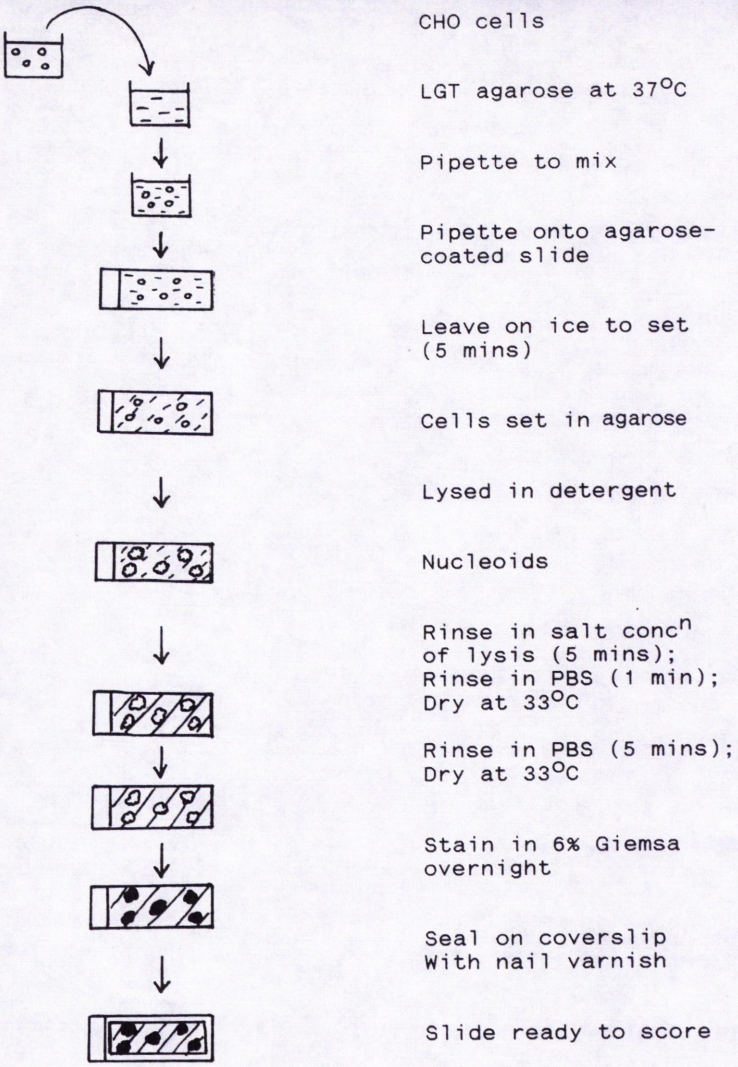


Figure 6.1 Nucleoid preparation method

3.1.2 Cell treatment before lysis

CHO cells were trypsinized, counted, diluted to $5x10^5$ - 10^6/ml in MEM and 2 ml aliquots of the cell solution per sample required were dispensed into 7 ml plastic Bijou bottles (Sterilin). The bottles were then gassed, sealed and kept on ice at all times from this point onwards to minimise repair. Samples were then irradiated on ice.

0.5 ml of the cell suspension was mixed with 0.75% low gelling temperature agarose (Sigma type XI, no. A-3038) at 37°C to make the final volume up to 5 ml and the cell concentration $5x10^4$ - 10^5/ml in the agar. Less than 1 ml of mixture was quickly pipetted onto the agarose-covered slides to give a thin layer and the slides were then put on ice for 5 minutes so that the agarose gelled. This time interval must be exactly the same for every sample if meaningful results are to be obtained. The cells were observed using a phase contrast microscope at this stage to check the cell number and agar thickness on each slide.

The initial 'priming' agarose layer did not stick well on all brands of microscope slides, tending to form a thick beady layer shrunk in from the edges which would not spread thinly across the entire slide and could easily be dislodged. The aforementioned slides were found to work well. A solution of LGT agar as used for the cell and agarose mixture also did not spread evenly, and cold agarose of either type did not stick to the slides well. The agarose-coated slides could be left to set for longer than one hour but if left for more than one day the agarose began to dry out, causing the background to be more grainy. It was important to ensure that the agarose/cell mixture was of optimum consistency when it was spread on the slide. If it was too warm the agarose would still be very fluid resulting in a thin layer with few cells per slide, and would drip off the slide, whereas if it were too cool it would form a thick layer on the slide with the cells distributed throughout the thickness of the agarose instead of all in the same plane.

This would result in uneven Giemsa staining across the depth of the slide (and staining differences between thick and thin layers) and more time consuming scoring due to much extra focussing.

3.1.3 Cell lysis

The slides were placed in a tray and gently covered with the lysis solution which contained either 0.5% triton X-100, 133mM Na_2EDTA, 10mM tris and 0.15 - 2M NaCl, or 2.5% SDS and 25mM Na_2EDTA. A higher EDTA concentration or NaCl could not be added to the SDS lysis solution as components of the solution would then precipitate out at room temperature. A high EDTA concentration was added as the lysis time required was long, so nuclease digestion of the DNA could be significant. The pH was set to either 7.4 or 9.5. The slides were then left for 90 minutes to allow the cells to lyse. When this was complete, the cells became invisible under the bright field microscope. The slides were rinsed in the salt concentration of their lysis solution, dried at 33°C, rinsed in PBS and dried again. The second rinse in PBS was found to reduce the amount of debris on the slide. The rinsing stages were found to be important as if cellular debris is left in the agarose it too is stained by the Giemsa, producing a distracting background. It was also necessary to filter the Giemsa otherwise large clumps became lodged in the agarose. It was important to lyse the cells as soon as possible after irradiation; results were found to be unpredictable if the cells were left for more than 30 minutes on ice before lysis, so the number of samples was therefore restricted to a maximum of 20 per experiment.

3.1.4 Staining

Slides were always dried before staining, as this was found to partially 'fix' the nucleoid structure and stick the agar firmly to the slide. Also if the slides were dried after staining, the remaining stain

on the slide would become very concentrated and the agarose would be more fragile and so more easily damaged.

After drying, the slides were rinsed in PBS to remove any residues in the agar such as salt crystals which had precipitated out of solution during drying and were then stained overnight in 6% Giemsa. This rinse before staining was not so essential when staining with ethidium bromide, but if the slide contained contaminating salt residues when it was put into Giemsa, the stain would form large clumps which would become fixed in the agarose, thus obscuring the nucleoids. After rinsing in PBS a coverslip was sealed onto each slide with nail varnish. This rinsing stage is important as large clumps of Giemsa tend to settle out of solution during staining and can become fixed in the agarose. The length of time for which the Giemsa-stained slides were preserved depended on the efficiency of the nail varnish seal - most samples lasted for at least 2 weeks and many for over a month. Alternatively, slides were stained for 45 minutes in Ethidium Bromide made up in PBS at concentrations ranging from 5 to 50 μg/ml.

'Stainsall' (BDH: Dahlberg *et al*, 1969) was also used. Slides were incubated overnight in 0.03 g/l Stainsall in equal quantities of formamide and distilled water. RNA stains bluish purple, DNA blue and protein red.

3.1.5 Scoring

The nucleoid dimensions were routinely scored using a Zeiss bright field microscope under x100 magnification with a focussing eyepiece and graticule. It was found that if the boundary of the centre was sharply in focus then the rest of the nucleoid including the halo would also be sharp. Any nucleoids which could not be focussed sharply, were overlapping others or which did not approximate to a spherical shape were not scored. The centre and total diameter were measured vertically and horizontally for each of 100 nucleoids per slide when scoring

Giemsa-stained slides; only 50 EB-stained nucleoids could be scored before the fluorescence began to fade.

3.2 Lysing of cells still attached to the culture flask.

The medium was removed from the flask and the cells were gently washed with PBS. The cells were then covered with lysis solution for 30 minutes; the lysis time was shorter than when in agarose as the solution does not have to diffuse through the agarose to reach the cells. Nucleoids were then rinsed with 1M NaCl then fixed in PBS containing 2.5% glutaraldehyde for one hour in preparation for scanning electron microscopy (see section 3.3). Care must be taken to treat the samples very gently to avoid dislodging the nucleoids from the flask surface, so these rinses were carried out by gently pipetting the solutions on and off the flask surface.

3.3 Other microscope techniques used

3.3.1 Fluorescence

Nucleoids were stained with ethidium bromide (5 - 50 μg/ml) for 45 minutes in the dark, then a coverslip was sealed onto the slide with nail varnish without rinsing off the stain solution. The slides were observed using a Zeiss or Leitz microscope. The fluorescence lasted for approximately 24 hours before fading. Re-staining was possible if the coverslip was not fixed on and the observation time was brief before soaking the coverslip off in PBS. If the coverslip were left on for too long out of solution then the agar was damaged on its removal.

3.3.2 Dark field

An inverted phase contrast microscope with the Hoffman frequency modulation system was used which gives an approximate dark field image by placing filters in the Fourier plane and on the field stop, half of

which are blocked out with black tape. This was used to directly observe cells lysing in solution without the need for stain and instead of using phase contrast where a halo effect is seen around every cell which may result in misleading results.

3.3.3 Electron microscopy

Various nucleoid preparation methods were tried for transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

For TEM a coverslip method was attempted. Nucleoids were prepared from CHO cells in the usual way except that an initial cell concentration of $2x10^6$ cells/ml was used and the agarose/cell mixture was pipetted onto 22 mm x 22 mm glass coverslips or 22 mm plastic tissue culture coverslips, both already primed with 0.1% agarose. The coverslips with prepared nucleoids were fixed in PBS containing 2.5% glutaraldehyde (30 minutes), washed in PBS containing 2% sucrose (3 changes), fixed in PBS containing 1% osmium (30 minutes) and washed again in PBS containing 4% sucrose (3 changes). The samples were then dehydrated using a series of alcohol solutions (50%: 10 mins; 70%: 10 mins; 90%: 10 mins; 100%: 3 changes of 20 minutes), followed by 3 changes of propylene oxide solution of 20 minutes each. After embedding in araldite (48 hours) the glass coverslips were snapped off the araldite block (containing the agarose layer) after cooling in liquid nitrogen. The samples must be handled very gently until embedding as the agarose would not stick well to any brand of coverslip tried and instead showed a tendency to float off in solution and become fragmented. All samples were then thick sectioned (including those prepared on plastic coverslips as these can be sectioned) and stained with methylene blue or Giemsa to locate the nucleoids. Had the techniques been successful the sections would then have been stained

with 1% uranyl acetate (stains mainly DNA) then 1% phosphotungstate (stains mainly protein).

To study cells lysed while attached to the culture flask with SEM, nucleoids rinsed with PBS were then fixed in 2.5% glutaraldehyde in PBS for one hour in the flask, and then partially dehydrated (10 minutes at 50% alcohol then into 70% alcohol). 10mm circles of the flask were stamped out using hot copper tubing straight into more 70% alcohol, then transferred to 90% alcohol for 10 minutes then to 100% alcohol overnight. This technique of stamping out discs from the flask was not used for TEM as the heat from the copper tube distorts and roughens the rim of the disc, thus making sectioning difficult.

A technique used for both TEM and SEM was to set a 2-3 mm layer of 2% bacto-agar in a petri dish then pipette the cell and LGT agarose mixture on top of this and allow to set at $4^{o}C$. Simply surrounding this with ice was not successful as the cooling effect did not reach the fluid agarose on the surface. Discs of bacto-agar with set agarose and cells on top were then cut out of the block with a scalpel blade and put straight into lysis solution, lysing for 90 minutes as normal. This avoided the problem of having to section the coverslip or break it off, but still allowed easy handling of the cells in agarose, the agar and agarose sticking to each other quite well.

3.3.4 Use of the quantimet

This is a computer-linked microscope which may be programmed to scan across a slide and measure and record various aspects of the morphology of the samples.

3.4 Nucleoid digestion by nucleases

DNase digestion of nucleoids was carried out with nucleoids both set in agar and lysed while attached to the culture flask. In both cases, cells were lysed as normal then washed with 1 M NaCl, followed by 3 washes in PBS containing 2 mM $MgCl_2$. DNase and RNase both require Mg^{++} as a cofactor - if EDTA is present it will chelate these ions and so inactivate the nuclease so, as EDTA is present in the lysis solution, this must be thoroughly washed out before DNase digestion. Nucleoids were then incubated in 5mM $MgCl_2$ and 200 ug/ml DNase I (Sigma) at 37°C for 60 minutes when set in agarose and for 30 minutes when lysed on the flask surface. The samples were then gently washed twice in PBS with 2mM $MgCl_2$, then twice in PBS only. Subsequent treatment (including SEM technique) was as for normal nucleoids.

3.5 Immunofluorescent staining of 67kD lamin protein

Nucleoids were prepared as normal in agarose on microscope slides and dried. After a brief rinse in PBS, 0.1ml of a 1:20 dilution of rabbit polyclonal antibody to 67kD lamin protein (from Professor RD Goldman) was pipetted onto the slide and a coverslip was gently lowered on top. The slide was then left in a humidified environment at 4°C overnight. The coverslip was soaked off in PBS and the slide given 2 washes of one hour each in PBS. If the DNA was also to be stained, the slide was then left in 50 μg/ml ethidium bromide for one hour at this stage then rinsed briefly in PBS. 0.1ml of 1 in 100 diluted FITC-labelled anti-rabbit antibody (Miles) was then pipetted onto the slide and left in a humidified environment at 4°C overnight. If EB staining is carried out on the same day as observation, even if using a very dilute concentration, the EB fluoresces so strongly, especially if added after the FITC stain, that the FITC does not show up. Also, to stain again after the FITC stain overnight, the coverslip must be well soaked off to prevent agarose

damage. Prolonged washing after the FITC stain did not alter the specificity of the stain but did reduce its intensity, so instead, slides were immersed in PBS for only 10 minutes prior to observation to reduce the background stain.

The FITC-labelled anti-rabbit antibody alone was also added to slides to check for non-specific binding to the nucleoids.

3.6 Electroporation of cells

This technique for producing holes in cell membranes (Knight, 1981; Knight and Scrutton, 1986; Sowers and Lieber, 1986) has recently been successfully used to allow the introduction of large molecules into cells (Winegar, 1988). CHO Cells were trypsinized and resuspended in PBS (with 0.02% trypsin inhibitor to halt the trypsin digestion and 5mM $MgCl_2$ to block the action of EDTA on the cell) then 0.5 ml cell suspension was pipetted into an electroporation chamber which was inserted into one of the compartments in the cell porator (BRL). $2x10^6$ cells were used, the capacitance being set to 800 μF respectively. If fewer cells than this are used, the cell survival is very low, while if a higher concentration is used the cells tend to fuse. The current was discharged at room temperature once the voltage had reached 260V, then the cells were immediately tipped out of the chamber into fresh medium and centrifuged at 1000 rpm for 5 minutes to remove cellular debris. Cells were resuspended in fresh medium and then nucleoids were prepared in the usual way.

Leaving cells on ice after electroporation is known to produce unpredictable results so cells were at room temperature before mixing with LGT agarose; the 5 minutes on ice to set the agarose could not be avoided.

3.7 Measurement of CHO cell diameters prior to lysis

Cells were set in DW agarose and left on ice for 5 minutes as is usual prior to lysis. The diameters of 6 cells were then quickly measured under the phase contrast microscope. More diameters were not measured on each slide as it was thought desirable to only measure these at the time after mixing with DW agarose that they would normally be put in lysis solution, in case the hypotonic solution was causing the cells to swell. This was repeated 5 times to obtain 30 cell diameter measurements.

4. Investigations into nucleoid morphology

The investigations into nucleoid morphology and the development of a nucleoid assay for DNA damage and repair were investigated concurrently but will be discussed in separate sections for clarity. The results of each assay will be considered independently here, then their implications to nucleoid structure will be discussed together in section 7.

4.1 Dark field microscopy

The optimum lysis time was deduced by observing cell lysis using a phase contrast microscope modified by the Hoffman frequency modification system to give a dark field image. Some cells were only completely lysed after 80 minutes, so a 90 minute lysis time was used.

Incompletely lysed cells could also be clearly distinguished as they stained very darkly after one hour in Giemsa, whereas fully lysed nucleoids could not yet be observed at this time.

4.2 Fluorescence microscopy - staining nucleoids with ethidium bromide

Ethidium bromide (Eth Br) was used (50µg/ml), a fluorescent dye which intercalates into the DNA. Although this technique gave good preparations, problems found were that the stain could not be preserved for any length of time, and was very faint after 24 hours even after staining and storing in the dark and that the fluorescent glare meant that it was difficult to see detail of the nucleoids. HeLa nucleoids have been shown to remain intact for a number of hours in solution, less so for chick nucleoids (Cook and Brazell, 1976b). These nucleoids set in agarose then dried down lasted much longer than this; when stored at 4°C the structure could be maintained for several weeks depending on the efficiency of the nail varnish seal to prevent dehydration. When Eth Br is exposed to light it gradually introduces breaks into the DNA by photoactivation, and when nucleoids are stained with ethidium bromide in solution the halo rapidly increases in size as a result. This was not found to occur in the nucleoids set in agar so dithiothreitol was not added to slow the photoactivation.

Eth Br was tried as all the successful methods published used fluorescent stains. The appearances of nucleoids lysed in triton 1M NaCl (DW agarose and PBS agarose), SDS (PBS agarose) and NLS (PBS agarose) are shown in Plates 4-6. All nucleoids showed a central region with a surrounding halo, the specific morphologies of which varied considerably.

SDS-lysed nucleoids had by far the largest halos. NLS-lysed nucleoids had small centres, and irregular halos. Their 'fuzzy' appearance made focussing difficult so they did not appear suitable for scoring damage by eye. Nucleoids from cells set in PBS agarose and lysed in triton and 1M NaCl were smaller and tended to be better defined than those resulting from SDS or NLS lysis.

Nucleoids from cells set in DW agarose and lysed in triton and 1M NaCl had larger centre diameters and smaller halos than when the cells were set in PBS agar prior to lysis, the central structures being symmetrical with well-defined centre boundaries and a far wider range of centre diameters were seen. The larger centres often had a more intensely stained inner central region. Here, with fluorescence, the central region could be studied by allowing the nucleoid stain to fade. Firstly, the outer halo faded, leaving the large central region. Then this large central area began to fade, leaving a more densely stained smaller central region, which was far better defined at this stage. After a considerable length of time, this centre faded in a 'granular' fashion.

For those nucleoids with just a small centre however, first the outer halo faded, then the inner region as a whole; no inner central structure was seen. Nucleoids set in DW agarose and lysed in triton and 2M NaCl had a similar morphology to those lysed in 1M NaCl as mentioned above. The bright staining of the complete structure with EB illustrates the presence of nucleic acid.

4.3 Bright field microscopy - staining nucleoids with Giemsa

It was decided to use a light stain in an attempt to avoid the problems associated with using a fluorescent stain. The stain should last as long as the preparation, a bright field microscope could be used and the 'fluorescent glare' would be avoided. Giemsa was selected as it had already been successfully used to stain nucleoids; Vogelstein *et al* (1980) found that nucleoids stained well in Giemsa but the drying part of the technique caused distortion. No distortion was found due to the drying stage of this technique; the nucleoid morphologies seemed the same as when staining with Eth Br. Examples of nucleoids prepared using the different lysis techniques are shown in Plates 4-6. The nucleoids showed the same morphologies as when stained with Eth Br although the images tended to be much sharper as is usual for a light stain compared with a fluorescent stain. Giemsa is a very non-specific stain but is unlikely to stain pure nucleic acids, rather indicating the presence of proteins in the nucleus, including those associated with chromatin if present.

4.4 Bright field microscopy - staining nucleoids with 'Stainsall'

'Stainsall' was used in an attempt to locate the proteins, RNA and DNA within the cell. All nucleoids used (SDS, triton 1M NaCl PBS; triton 1M NaCl DW) showed the same morphology as those stained with Giemsa or Eth Br and stained blue all over - the 'DNA colour', with no evidence of purple (RNA) or red (protein) staining. This implies that the majority of the material in the centre and halo is DNA. Unfortunately the stain was faint and faded quickly so attempts to photograph these preparations were unsuccessful.

4.5 Scanning Electron Microscopy

Nucleoids have mainly been studied by electron microscopy (EM) using Kleinschmidt's procedure (1969), which involves the spreading of the nucleoids at an oil/water interface and subsequent shadowing and observation by scanning electron microscopy (SEM). What was required here, however, was a technique to observe nucleoid structure as close as possible to that seen in agar in the unfixed state - this would not be maintained if the nucleoid were spread; Cook (1984) produced halos from HeLa nucleoids after spreading, but not otherwise. Also, the nucleoids in agar appear to have a slightly 3-dimensional structure - spreading would not demonstrate this.

SEM was used first. This technique will only analyse surfaces of structures, so if nucleoids were set within an agarose layer, just the surface of the agarose should be seen and not the nucleoids within. It was still decided to first use SEM to look at cells set in agarose, as firstly agarose was known to slowly dissolve when in alcohol, so it was thought that leaving the agarose in 75% alcohol overnight may etch away the agarose surface to reveal the nucleoids. Secondly, during the critical point drying stage of preparation, the temperature briefly reaches approximately 60^{o}C and it was possible that this may melt the surface of the agarose enough to see the nucleoids below.

As is shown in Plate 7a, some regions of the surface were very bumpy, presumably due to the nucleoids still being below the surface. In others however, structures of similar dimensions to nucleoids and of a similar density to that of the known cell concentration were seen on the surface (Plate 7b) but did not have the same structure and in some cases appeared virtually crystalline, giving rise to the suspicion that these halos were simply artefacts, possibly due to the crystallization of residual salt from the lysis solution, or PBS in which the glutaraldehyde was made up, possibly precipitating at a site on the agarose with a nucleoid just under the surface.

It was then decided to lyse the cells while still attached to the culture dish, to eliminate the problems found with agarose. Vogelstein *et al* (1980) lysed cells growing attached to a coverslip and found that the resulting nucleoids remained attached, but they had only observed these using a fluorescent stain. SEM micrographs of fibroblasts attached to the culture flask surface before lysis are shown in Plates 1 and 2 and nucleoids after lysis are shown in Plates 8 and 9. The nucleoid preparations were difficult to observe in great detail or photograph as they were so thin that little contrast could be seen. The kV was kept much lower than for attached fibroblasts, otherwise the beam passes straight through the sample, which becomes transparent. An outline of the former cellular boundary was seen, and in addition there appeared to be fibres of some kind forming a halo around the centre of the approximate diameter of the halo seen in light microscopy. It is a possibility that these fibres are composed of DNA as they bear similarities to the rather thickened fibres surrounding spread nucleoids which were thought to be DNA which had condensed during alcohol dehydration (McCready *et al*, 1979).

4.6 Transmission electron microscopy

SEM provided some interesting information on nucleoid structure but not very much on the fine detail, or of the substructure maintaining their conformation. TEM would give important information on the structure of nucleoids if serial thin sections could be made. TEM of sectioned nucleoids has been successfully carried out (Jackson, 1986), resulting in a rather delicate image. The aim was to fix, embed, section and stain the agarose containing nucleoids, and observe them using the TEM. Unfortunately, no nucleoids could be found in preliminary thick sections when staining with Giemsa (stains nucleic acids and proteins) or Methylene blue (stains proteins) after any of the preparation methods tried (see Materials and Methods section 3.3). The two possible reasons

are firstly that the treatment is too aggressive and the nucleoid structure is destroyed by the changes in osmolarity and nature of the solution or secondly the nucleoids have been well preserved but the staining technique is not showing their structure. The second option seemed the most likely as nucleoids prepared satisfactorily for SEM using the same initial glutaraldehyde fixing protocol; once the samples were fixed at this stage it is unlikely that later steps could completely destroy the structure.

4.7. Bright field and scanning electron microscopy - digesting nucleoids with DNase

Nuclease digestion of nucleoids was used to gain information on nucleoid structure. The structures did not stain with ethidium bromide, implying that virtually all the DNA has been removed, but Giemsa did stain the structures faintly as is shown in Plate 10. A large amount of background contamination is visible as a high contrast was needed during developing the print to show up the residual structure. DNase digestion removes the outer halos of the triton-lysed nucleoids leaving central regions, none of which were found to have a diameter as large as the larger central regions in intact nucleoids (Plate 3).

Nucleoids were also prepared from cells attached to the culture flask surface then DNase treated and observed under the SEM. The fixation drastically altered the remaining structure (Plate 11), presumably because the residues were fragile and could not survive the procedure, the structures seen bearing little resemblance to the DNase treated structures seen under bright field microscopy and stained with Giemsa. The nature of the fibres emanating from the nucleoid are therefore still not known.

Nuclease digestion of SDS-lysed nucleoids left nothing visible when stained with Giemsa, Eth Br or SEM.

4.8 Fluorescence microscopy - immunoassay to locate the nuclear lamina

Nucleoids were treated with a polyclonal rabbit antibody to the 67 kD lamin protein (lamin B) then stained both with FITC (fluorescein isothiocyanate)-labelled anti-rabbit polyclonal antibody which should locate this lamin protein, and with Eth Br which should locate the nucleic acids. It was thought important not to just study the location of the lamins in isolation but rather in connection with the rest of the nucleoid structure, so nucleoids were also stained with EB which should locate the nucleic acids. Resulting nucleoids are shown in Plate 12. FITC fluoresces green but is far less intense and fades much more quickly than EB which made the study and photography of the two stains difficult. The exposure time was not more than 30 seconds, as after this time the FITC had faded so only the Eth Br fluorescence would be detected. Even so, the FITC-labelled regions do not appear green in the plates but rather yellow, although their presence can be clearly distinguished in these plates from those just stained with Eth Br (Plate 4).

These experiments did show that the lamins are located in a central region within the area here termed the centre of the nucleoids; where there was variation in the centre diameter of the nucleoid, the diameter of the lamina was unchanged.

4.9 Fluorescence microscopy - effect of varying the ethidium bromide concentration on the dimensions of triton DW nucleoids.

This is the classic technique to demonstrate the presence of loops of DNA (Cook and Brazell, 1975; 1976) and was used here to check whether the dose effect was due to a DNA phenomenon and not other cellular effects.

The effect of varying the ethidium bromide (Eth Br) concentration on nucleoid dimensions is illustrated in Figure 6.2. By measuring centre

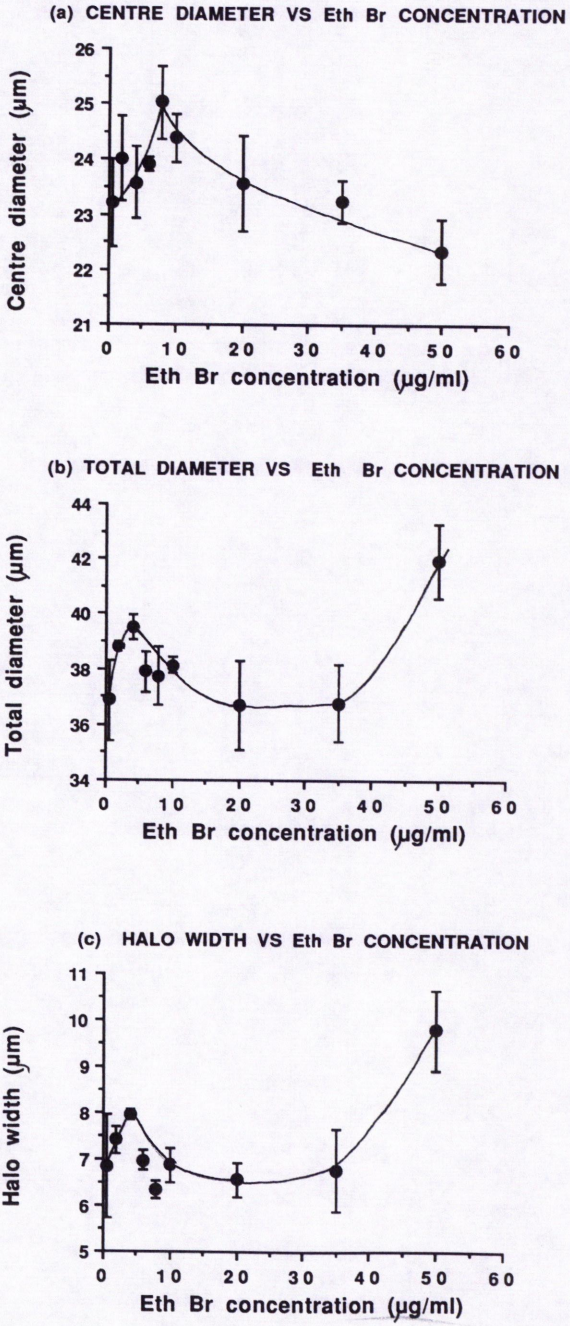


Figure 6.2 The effect of varying the concentration of ethidium bromide stain on the dimensions of nucleoids produced by mixing in DW agar and lysing in triton. Results of 2 experiments are shown

diameter, total diameter or halo width, a peak in the diameter was seen between 0 and 10 μg/ml Eth Br. The peak was most pronounced when measuring the centre diameter, where a true biphasic curve was obtained, peaking at approximately 8μg/ml Eth Br; when measuring total diameter or halo width, the low Eth Br concentration peaks were smaller and at high Eth Br concentrations an increase in size and uncertainty was seen.

A biphasic curve such as this suggests that one is dealing with loops of DNA; however, this observation was unexpected as the slides had been dried down before staining; DNA would not be expected to survive this treatment intact and able to respond to Eth Br. The mean diameters of the 0 Gy Giemsa stained nucleoid centres corresponded to the peak sizes of those Eth Br stained, so the irradiated nucleoid centre diameters were greater than the peak Eth Br diameter (see section 6.1). Attempts to stain with both Eth Br and Giemsa were unsuccessful; whichever was added first was taken up strongly but the second hardly stained at all. When both stains were used mixed together, a faint outline could be seen from the EB, but nothing of the Giemsa.

4.10 Cell diameter measurement before lysis

The diameters of cells set in DW agarose were measured at the time when they would have been put in lysis solution to determine whether this diameter bore any relation to the centre diameter of nucleoids set in this agarose before lysis. This distribution of cell diameters obtained is shown in Figure 6.3. The mean diameter was found to be 17μm +/-3μm, although the distribution was clearly skewed.

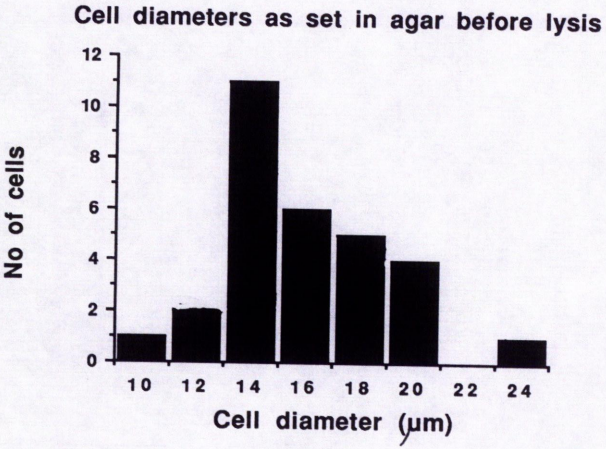


Figure 6.3 Distribution of cell diameters set in agar before lysis

5. Comparison of the response of different lysis techniques to X-irradiation by scoring nucleoid dimensions

5.1 Preliminary nucleoid scoring

All published techniques for direct observation of nucleoids use fluorescent stains but it was decided here to try to develop a technique staining with Giemsa instead. The advantages of this would be that a conventional light microscope could be used and the stain should last as long as the nucleoid preparation. Additionally, the computer-linked microscope could only be connected to a bright field microscope, the development of an automatic technique being an aim of this study. Fluorescently stained nucleoids also could not be scored by eye with a graticule without difficulty due to the dark background.

Only a limited amount of information could be gained from simply looking at the change in appearance of nucleoids after X-irradiation; measurements must be taken to be certain whether any particular preparations would give a dose-response.

To score these changes in dimensions the total diameter and the centre diameter of each nucleoid were measured vertically and horizontally using a graticule in the eyepiece of the microscope and the mean of each was calculated. From these measurements the halo width was deduced. 100 nucleoids were scored in this way on each slide. The triton lysed samples with 0 M and 0.15 M salt samples were difficult to score so these were not included when deciding which nucleoid preparation to use but one slide of each was scored for completeness. At least two independently prepared slides of 100 nucleoids were scored for every other preparation.

It was thought that the clearest way to represent the data would be to either determine the mean or the median of all values of centre

diameter, total diameter and halo width for each sample. The median is the value that falls in the middle when the data is sorted in order of magnitude. The mean is the average of all the values. The advantage of using the median is that it is more resistant to distortion by a few outlier points than the mean (for example, if a few quadruploid cells are present with far greater centres and/or halos), or due to the population not approximating to a normal distribution. The main disadvantage of using the median is that error estimates are far more difficult to determine than when using mean values - standard error of the mean (SEM) *etc* only hold for errors about the mean. It is important to have good error estimates for data so the distributions of nucleoid dimensions were analyzed to determine whether they were close enough to normal distributions for the means to be used. If a distribution is not normal but is assumed to be so, calculated estimates of uncertainties will be larger than when working with a normal distribution and not strictly valid, and the mean will not be a meaningful description of the data.

Histograms showing the distribution of dimensions of nucleoids of irradiated (3 Gy) and unirradiated cells set in DW agarose and lysed in 1M salt and the square root of all these dimensions are shown in Figures 6.4 and 6.5. Most nucleoid distributions appear rather positively skewed, that is, the mode (*ie* the value which occurs most frequently, the peak in the histogram) is less than the mean but it is difficult to determine just by looking at a histogram whether the distribution is too asymmetric to be an approximation to a normal distribution. This is not unexpected; cell-cycle-dependent size distribitions are skewed towards larger diameters, and the distribution of nucleoid halo sizes as measured by direct observation have been shown to be similarly skewed (Roti Roti and Wright, 1987).

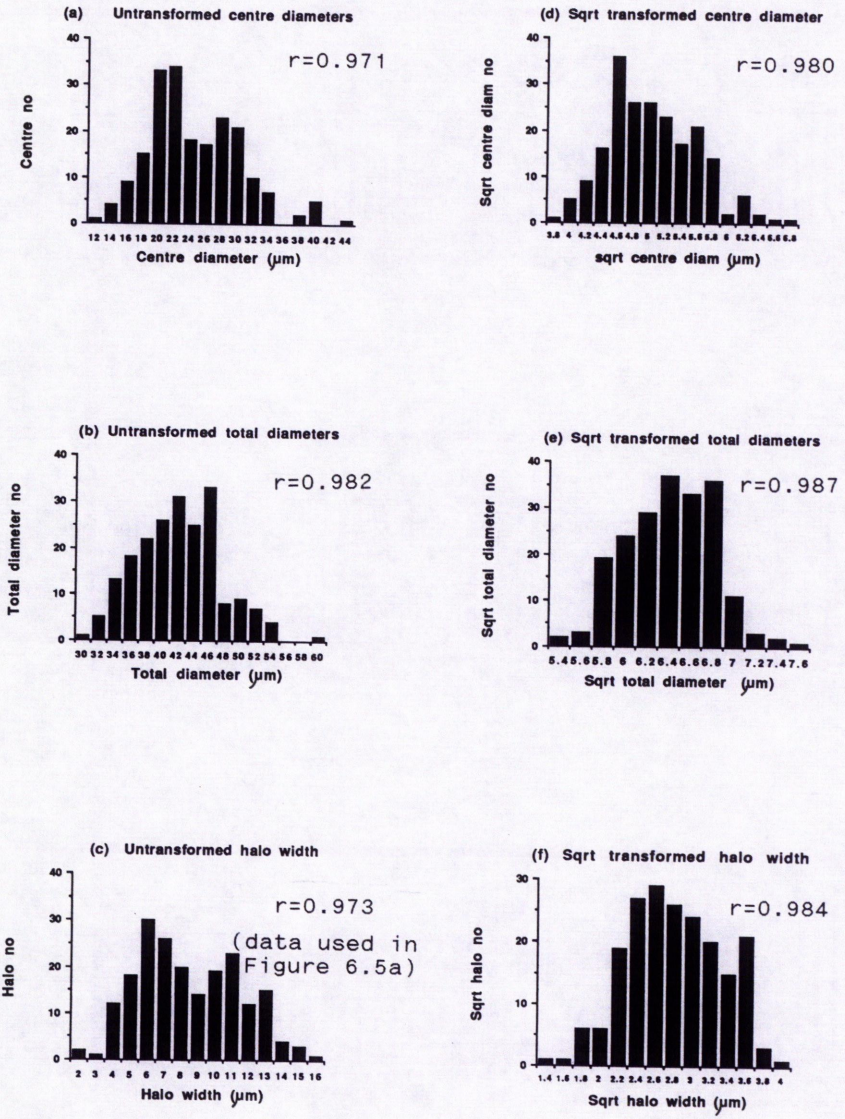


Figure 6.4 Histograms showing typical distributions of nucleoid dimensions prepared from cells irradiated with 3Gy and using untransformed and square root transformed data. Results of 2 experiments scoring 100 nucleoids in each experiment shown.

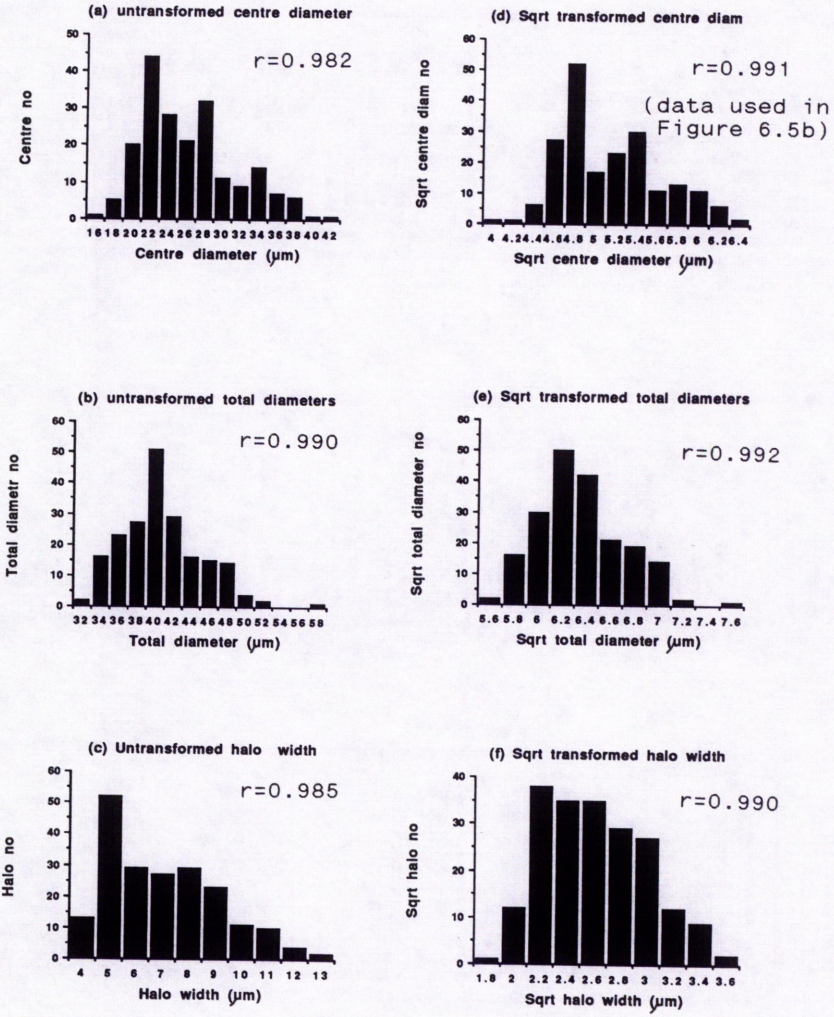


Figure 6.5 Histograms showing typical distributions of nucleoid dimensions prepared from unirradiated cells and using untransformed and square root transformed data. Results of 2 experiments scoring 100 nucleoids in each experiment shown.

5.2 Determination of the best transformation to use for the comparison

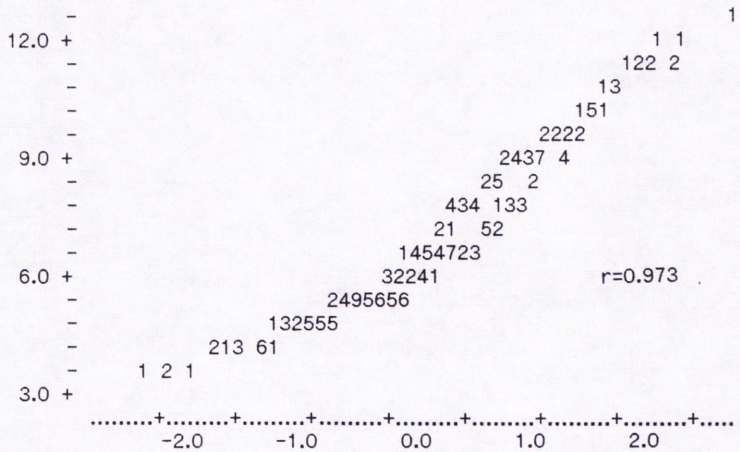
If a distribution is not close to normal, it is quite correct, and advantageous, to transform the data to make it more normal - that is, to multiply each data value by a common factor. The most common transformations are, in order of strength: square root, $\log_e$ (ln) and negative reciprocal ($-1/x$).

First a normal probability plot was obtained for each distribution (*ie* histogram of measurements). This is a plot of each measurement within the sample against the measurements one would get on average if the sample was from a normal population but with the same mean, SD and number of measurements as seen for the sample. A straight line results if the samples are from a normal population; if not, the line is curved. Examples of such plots are shown in Figure 6.6. Where lines were found to be completely straight or very curved it was straightforward to classify the data as normal or not, but most lines were slightly curved which made the interpretation more difficult. The line in Figure 6.6a is more curved than that in Figure 6.6b, but it is difficult to deduce from this how much the sets of data deviate from normal distributions. The Pearson product moment correlation coefficient, denoted r, was then calculated for each distribution. This involves the analysis of the deviation of the points on the curve from their mean, generating the coefficient r as follows:-

$$r = \mathrm{Corr}(X,Y) = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{(x-\bar{x})^2 (y-\bar{y})^2}}$$

In this situation, the closer r is to +1, the more the two sets of points on the plots are associated and so the closer the nucleoid data

(a) Untransformed halo width data after irradiation with 3 Gy



(b) Square root transformed centre diameter data - unirradiated

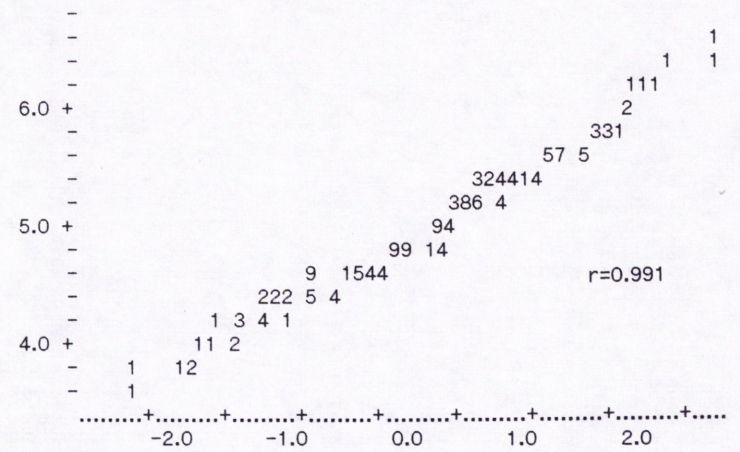


Figure 6.6 Normal probability plots of 2 sets of nucleoid data (from Figures 6.4 and 6.5)

is to a normal distribution. Values of r obtained for each sample using untransformed data are given in Table 6.2. These values may be compared with those published in tables (*eg* Filliben, 1975) to determine how close to a normal population the data is.

In statistical terms, the hypothesis to be tested is that the data approximates to a normal distribution. This is termed the null hypothesis and is denoted by H_o. To carry out a quantitative study into the normality of the data, the significance level of the statistical test must be stated; this represents the probability of making a type I error. A type I error occurs when the null hypothesis is rejected when it is actually true. In this situation, a type I error would be the rejection of the hypothesis that the distribution is normal when the distribution was in fact normal. If the data can still be classed as normal when a high significance level is set, then it must be very close to a normal distribution.

Here, the significance level is set, then the value of r for this significance level and the known number of samples is read from tables (Filliben, 1975); then r is determined for the experimental data. If the experimental r is greater than or equal to the value of r from the tables, then the null hypothesis is accepted at the significance level set and the data is assumed to approximate to a normal distribution. It is important to remember that the hypothesis of normality is never totally accepted or ruled out, but just rejected at this particular significance level. A value of r close to 1 suggests that the sample approximates to a normal population; how close r must be to 1 for the population to be accepted as normal depends on the significance level set for the test. As well as being the probability of making a type I error, the significance level is the probability that the data might have arisen by chance. In most experiments a 5% significance level is

set; (values of r greater than or equal to 0.981 in this case). This means that if the result of the test is that the data can be classed as a normal distribution, there is a 5% chance that the data is not in fact normal and this distribution simply arose by chance. A 1% significance level was also set here to determine how close the data was to a normal distribution at a more stringent significance level, (values of r equal to or over 0.987).

The values of r obtained for each lysis treatment and transformation are given in Tables 6.2 to 6.5. Those experimental values of r which are nearer to +1 than r from tables corresponding to the 5% and 1% significance levels are indicated by underlined characters and bold underlined characters respectively. The data is summarized in Table 6.6. Table 6.2 shows that around half of the untransformed data is below the value of r for a 5% significance level (0.981); this data would still be just usable untransformed, but it would be more correct to use a transformation to bring the distributions closer to normal. There are clearly significant differences in the deviations from normality of the different distributions. At this stage however it is simply required to select the transformation which will give the best overall approximation to normality to compare all curves with each other. *ie* if $-1/x$ were used, this would give approximately normal distributions for centre and total diameter data but the halo curves would generally be very poorly approximated to normality. From Table 6.6 it appears that the ln transformation would be the best to use to compare all the lysis methods with each other. This is clearly not the best in each individual case, but overall produces the least deviations from normality.

Table 6.6 Preliminary experiments - overall mean values of r for all treatments.

	UNTRANSF	SQRT	LOG_e	$-1/x$
CENTRE	0.962	0.974	0.982	0.988
TOT DIAM	0.974	0.980	0.984	0.989
HALO	0.982	0.982	0.982	0.955
MEAN	0.973	0.979	**0.983**	0.977

5.3 Comparison of different lysis techniques using the $\log_e$ transformation

Different lysis solutions produced distributions of their dimensions for which various different transformations brought them to a normal distribution; no one transformation brought all the data to normality as different cell treatments produced different shaped distributions. In addition, the irradiated nucleoids gave a more skewed curve than unirradiated. For the triton-lysed cells, all transformations were an improvement on the untransformed measurement, but the best halo transformation did not correspond to the best centre and total diameter transformation; the square root transformation seemed the best overall. For the SDS-lysed cells however, the negative reciprocal transformation seemed the most suitable, and as r became progressively higher through the three transformations, possibly an even stronger transformation would be best.

The mean and SD of each data set's ln transformed centre, total diameter and halo measurements are shown in Table 6.7. The only data set showing a clear dose-effect was the centre diameter of cells set in DW agarose and lysed in triton and 1M NaCl.

It was decided to further investigate this lysis method as it was an observation not previously reported and so a study of its viability as an assay would be worthwhile. Also, as the changing centre diameter gives a dose effect curve, which has a for more definite boundary than the halo, this could be more accurate to score and also easier to automate.

5.4 Determination of the best transformation to use to study Triton 1M NaCl DW nucleoids

The values of r for different transformed distributions are shown in Table 6.8.

Table 6.8 Values of r for different transformed Triton 1M NaCl DW agarose measurements, both unirradiated and after 3 Gy

	X	SQRT X	LN X	-1/X
CENTRE	0.977	0.983	0.985	0.969
TOTAL DIAMETER	0.978	0.984	0.987	0.986
HALO	0.982	0.986	0.977	0.918
OVERALL	0.979	**0.984**	0.983	0.958

Table gives means of Triton 1M NaCl nucleoid centre, total diameter and halo parameters, after various transformations of the data.

Underlined figures - transformation giving the highest value of r for each nucleoid dimension.

Bold underlined figure - transformation giving the highest value of r overall.

It does appear that, to be able to compare the effects of irradiation on the halo as well as on the centre and total diameter, the square root transformation would be better to use here as the value of r is most consistent for all 3 measurements; none of them give a very low score. This is because the distribution of halo sizes is less skew than the distribution of centre of total diameter sizes. This is due to the occasional very large centres seen. The ln transformation gave the highest values of r for the centre and total diameter distributions, but

r for the halo distribution was low, so this transformation would not be so useful to compare all 3 parameters. The untransformed and $-1/x$ transformed distributions did not give high values of r and so were not considered. The square root transformation to a normal distribution is most easily explained statistically, as this implies that the data approximates to a Poisson distribution. This distribution is often found in biology where the data consists of integers arising from counts of objects, such as the number of bacteria per plate or the number of chromosomal aberrations per cell and also in the measurement of cell diameters in an asynchronous population (Figure 6.2). There was however little to choose from between the ln and square root transformations.

The irradiated nucleoid distributions were found to be more skew than those unirradiated.

5.5 Use of the quantimet

As the dimensions of a definite central region were measured to score the damage of nucleoids rather than of a fuzzy halo, this technique seemed well suited to an automatic scoring method. In principle, the method should have worked well, but problems arose which could not be overcome. The system was set to auto focus on the first 3 nucleoids found while scanning across the slide, and record the mean light intensity of the centres and halos and limits of uncertainty. These intensities were stored in the memory of the system. The system was then programmed to scan across the slide, auto focussing on each nucleoid and measuring the area of each pre-set intensity within each nucleoid to be scored, then to compute the area of the centre as a percentage of the total halo and centre area. This was thought to be more sensitive to any changes in nucleoid parameters than the usual method, as the centre diameter should increase and the halo diameter

should decrease with increasing X-ray dose, so this was used for the test run. The first problem was that of staining the nucleoids. The quantimet could not be used with fluorescent stains, so Giemsa was used. The darkness of stain varied from one nucleoid to another; this was not very obvious when scoring the nucleoids visually, but was significant when scoring with the quantimet. This was initially thought to be due to nucleoids at different levels throughout the agar taking up different amounts of Giemsa during the staining period - as Giemsa consists of large molecules, these may take a long time to diffuse through the agar and so those nucleoids at the bottom of the layer would stain less intensely than those at the top. However, when staining nucleoid preparations with EB, a far smaller molecule which stains the slides fully after 45 minutes and is quantitative depending on the amount of DNA present, such staining irregularities are still seen (albeit to a smaller degree) even after staining for 24 hours. It therefore did not seem worth persevering with a true quantitative light stain such as Feulgen's if the same staining differences would be seen.

The boundary which the quantimet set for the centre and halo was shown on the display screen. The system was programmed to score 100 nucleoids and its scoring of each nucleoid was observed. A major problem with the scoring was quickly identified. As well as there being different intensities of stain from one nucleoid to another, the staining is also not uniform within each nucleoid, the centre being more darkly stained near the middle and the halo more darkly stained nearer the centre. Again, these differences are very difficult to distinguish between with the naked eye, but are made evident when using the quantimet. The halo also does not stain in a uniform way, but has a grainy appearance, being made up of many darker dots. The small centred nucleoids were the most darkly stained, and the large centred ones more

diffuse. When a very darkly stained nucleoid is being scored, rather than defining the boundary of the centre at its true limit, the system will also include dots in the most central region of the halo. Conversely, when a paler stained nucleoid is being scored, the boundary of the centre will be set inside the true limit, both resulting in an irregular periphery to the area scored. A similar effect was seen when scoring the boundary of the outer halo. The system can be programmed to smooth the irregular outline but not to set the centre and halo boundaries to their true limits in every case. This meant that the mean centre and halo diameters scored would not be representative of the actual measurements. 100 nucleoids were scored for a 0 Gy and a 3 Gy slide which had already been scored by eye and found to show the normal dose response. The results of the quantimet scoring are shown in Figure 6.8. Unexpectedly, the centre diameter as scored using the quantimet was found to <u>decrease</u> after irradiation. On analyzing the scoring of each nucleoid it was found that in the 3 Gy samples, even though the mean centre diameters were larger, these larger centres were generally paler stained, so the diameter measured by the quantimet was well within the true diameter. This is an interesting observation in itself; even though Giemsa is not a quantitative stain, it appears semi-quantitative and this effect may imply that the same amount of material is present in unirradiated and irradiated centres and in the irradiated centres the material is more spread out. Even if this did give a consistent dose-response, it could not be extrapolated back to a physiological explanation and did not seem worth pursuing.

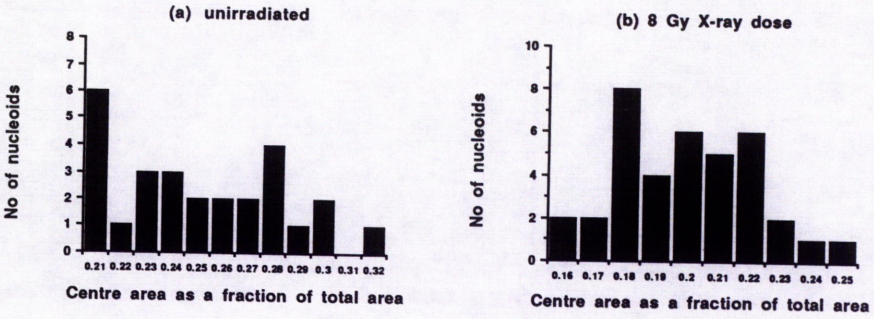


Figure 6.8 Use of the quantimet to score nucleoid dimensions

7. Use of the new nucleoid technique to study DNA damage and the effect of trypsin and electroporation on nucleoid structure

7.1 Radiation dose-response

Figure 6.9 shows the nucleoid dose-effect curves determined by measuring the mean centre diameter, total diameter and halo width, plotting the untransformed data and the square root transformed data. The centre diameter clearly gives the best dose-effect; the diameter increased from 0 to 4 Gy, then is essentially independent of dose from 4 to 12 Gy. The total diameter and halo width both showed a general decrease in size with increasing dose, but this was no more than a trend and could not be used as a dose-effect relationship. The statistical analysis predicted that the square root transformation produced the best approximation to a normal distribution from the data; clearly this transformation gives a linear dose response from 0 to 4 Gy with slightly smaller standard error of mean, while the response from 0 to 4Gy using untransformed centre distribution data was rather curved with slightly larger standard error of mean.

Slides scored immediately after staining and which had not dried out after one month at 4^{o}C showed reproducible scoring.

7.2 Repair kinetics

The nucleoid repair kinetics after X-ray doses of 4 Gy and 12 Gy are shown in Figure 6.10. The kinetics still show large uncertainties but a general decrease in centre diameter with time of incubation at 37^{o}C was seen in both cases. More repeats were not carried out as the technique was not found to be useful to monitor repair after trypsinization. Further experiments would provide more accurate kinetics.

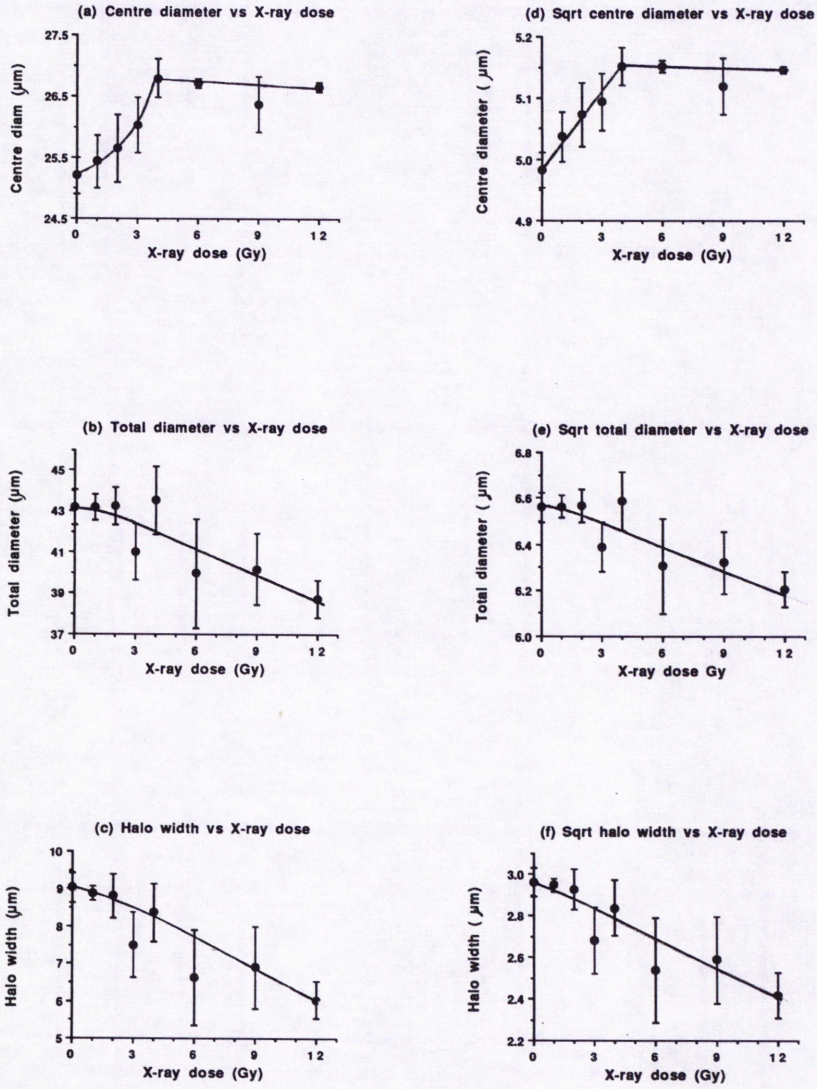


Figure 6.9 Nucleoid dose-effect curves using untransformed and square root transformed data. Each point represents the combined results of 5 to 7 experiments.

One illustration of best-fit curves to the points is shown. Nucleoid diameters of 26 µm would be measured as 54.5 units on the graticule to an accuracy of 0.5 units, resulting in an error in measurement of 0.2 µm.

(a) 4 Gy

(b) 12 Gy

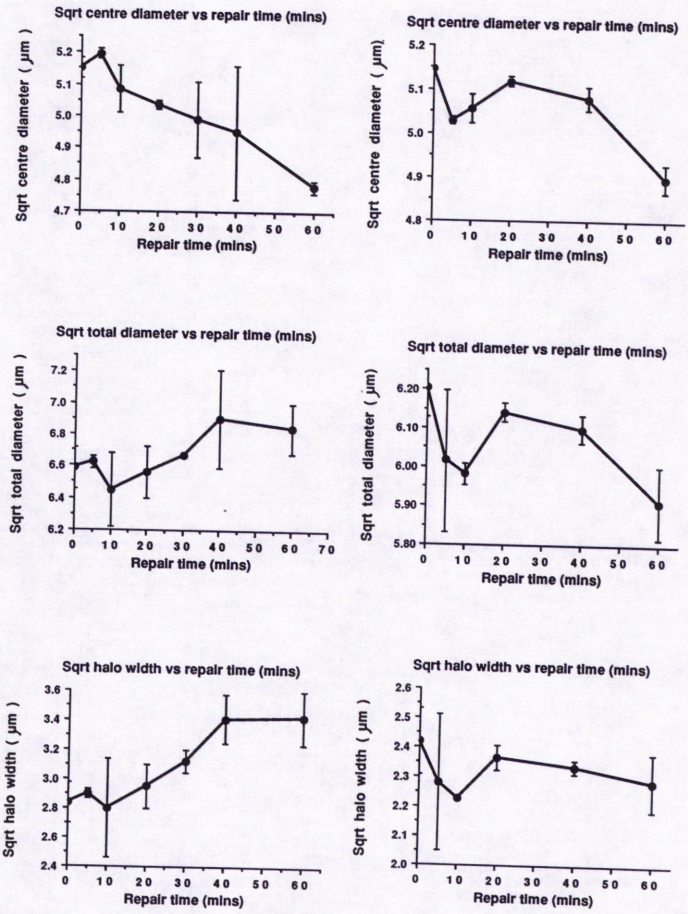


Figure 6.10 Nucleoid repair kinetics using square root transformed data. Each point represents the combined results of 2 experiments.

7.3 Electroporation

Nucleoids derived from electroporated cells are shown in Plates 13 and 14. A wide range of nucleoid morphologies was seen, some (the minority) being normal but many were altered in some way. Some showed a total loss of halo (Plate 14) but the most common structure was as shown in Plate 13. with one (or, less commonly, more than one) clear differentially staining channel passing through the structure. As it was not possible to score the porated samples, the intended studies of porating in restriction endonucleases and trypsin were not pursued.

7.4 Effect of trypsin on nucleoid structure

The aforementioned dose-effect curves and repair kinetics were carried out using cells trypsinized for 5 minutes to obtain a cell suspension; the initial aim here was to repeat the experiments with a 15 minute trypsin incubation. Typical (0 Gy) nucleoid morphologies are shown in Plates 15 and 16. Nucleoid structure is clearly altered here, but in a different way from after electroporation. No bands crossing the nucleoid were seen but the halo structure appeared less organized. Again it was not found to be possible to score these slides so the dose-effect curve and repair kinetics after 15 minute trypsinization were not investigated.

7. Discussion

This chapter considers the development of a nucleoid assay and also illustrates the associated difficulties. Nucleoid preparation using CHO cells had not been reported before, nor had the SEM technique been developed. Also no reports of SEM of unlysed CHO cells could be found in the literature, so this may not have been done before.

The parameter under investigation did give a dose-effect, but it is important to then consider possible mechanisms behind these observations.

7.1 Time of lysis

The lysis time to ensure all cells had lysed was 90 minutes. This is very long compared with previously published techniques (Cook and Brazell (1975) lysed for 15 minutes before sedimentation) but these nucleoid sedimentation methods were carried out in solution so no diffusion problems occured. This is still longer than the 30 minutes used by Cook and co-workers for cells set in agar beads (Cook and Brazell, 1975a; 1975b; 1976) - possibly the beads are so fine that the lysis solution can quickly diffuse through to the cells.

7.2 Setting cells in DW or PBS agar before triton lysis

A larger centre diameter of DW agarose nucleoids was seen compared with those nucleoids set in PBS agarose. This was initially thought to be due to the hypotonic shock swelling the nuclear cage, but the immunocytochemical experiments showed the lamina to be located within this central region and to have a fixed diameter independent of the centre size.

Hypotonic buffers have been used to isolate nuclei from cells, but the complete cell was still present here just before lysis as determined by phase contrast microscopy. Hypotonic buffers are also known to activate nucleases which degrade the DNA (cited in McCready *et al*, 1982) so this may also be happening here; possibly the outer halo is heavily damaged DNA from the action of these nucleases. Williams and Little (1974) showed that 5 minutes of hypotonic shock to cells resulted in DNA degradation within one hour.

Cells lysed without salt resulted in nucleoids with no halos, the central region being of a similar size to the PBS-lysed nucleoid centre and was never as large as the DW-lysed nucleoid centre.

7.3 Staining nucleoids with ethidium bromide

Nucleoids stained well with ethidium bromide (Eth Br) indicating the presence of nucleic acids in the centre and halo. The image was quite stable, and no rapid expansion in any part of the nucleoid was seen due to breaks being rapidly introduced into the DNA by photoactivation as is routinely seen when observing nucleoids in solution. Cook and Brazell (1976b) found that after 15 minutes' observation of Eth Br stained nucleoids, the intensity of fluorescence had diminished but extended over a larger area. One explanation is that the UV source used was weak and did not penetrate the agar very efficiently so the damage incurred in the DNA was minimal. If this were correct, then setting cells in agar before nucleoid production could be an advantage if direct scoring of Eth Br stained nucleoids was to be carried out. Alternatively, if the outer halo were not DNA but RNA and protein then it would not be expected to 'explode' in this way as it would not be supercoiled. Another possibility is that the outer halo is DNA but the drying down of the slides before staining destroys the

supercoiling, stabilizes the structure and so prevents this expansion. Evidence against this idea is that increasing concentrations of Eth Br still induce a small biphasic size response in the central region when slides of nucleoids are rehydrated and stained; this would not be possible if drying down completely fixes and degrades the structure. The presence of a biphasic response to Eth Br when studying *dried down* nucleoids was unexpected; a biphasic curve as seen in the EB experiment suggests that one is dealing with intact loops of DNA. Nucleoids prepared in 1M NaCl have been shown to remove the DNA supercoiling at an Eth Br concentration of 4 μg/ml (Cook and Brazell, 1976; Cook *et al*, 1976) and at 2-5 μg/ml (Vogelstein *et al*, 1980). The centre diameter gave the clearest peak here, which is somewhat higher at 8 μg/ml, whereas the less distinct peak in the halo width was seen at 4 μg/ml.

A possible explanation of the presence of this response is that DNA loops are present but are trapped within the hole in the agarose made when the cell was set in the agarose before lysis, but Eth Br can still intercalate and cause the loops to expand within this space. As agarose is slightly flexible, this could result in a slight expansion in the diameter of the hole. A biphasic response to Eth Br was not seen for the outer halo; as it stains with Eth Br it must contain DNA or RNA; the results of the Stainsall experiments (section *7.5*) suggest that the halo is DNA, and as such should also give a biphasic response. Possibly this is DNA which is heavily damaged and so relaxed and would not respond to Eth Br. Alternatively, the Eth Br could be staining RNA in the halo, DNA not being present.

7.4 Staining nucleoids with NLS and SDS

Nucleoids lysed in NLS had a very irregular, fuzzy structure and small centres, and looked far less uniform than those nucleoids produced

from SDS lysis. This may be connected with the effect seen in neutral elution where NLS lysis gives a far more sensitive dose-effect curve, possibly due to differences in the lysis action between NLS and SDS.

DW or PBS agarose made no difference to the NLS or PBS-lysed cell nucleoids, which did not support the hypothesis that setting a cell in DW agarose caused the cell to swell and so leave a larger hole in which the DNA could spread out at lysis.

7.5 Staining nucleoids with Stainsall

Stainsall staining showed the complete nucleoid structure the DNA blue colour, and no part stained purple (RNA) or red (protein). This implies that DNA is a major constituent of the centre and halo, masking the RNA and protein staining.

7.6 Staining nucleoids with Giemsa

Another unexpected effect was that the mean sizes of the 0 Gy Giemsa stained nucleoid centres corresponded to the peak sizes of those EB stained. This would initially seem to imply that if loops exist within the centre after Giemsa staining then they are at their maximum extended state, but this cannot be so as the mean centre diameter of the Giemsa-stained nucleoids expands still further after X-irradiation; Giemsa must interact with the nucleoids in a different way from that of EB. The outer halo however was smaller in the EB stained nucleoids than for Giemsa. Giemsa would not stain pure DNA so the outer halo in SDS-lysed nucleoids must consist of a significant amount of protein; SDS lysis removes almost all protein from chromatin and so the remaining DNA should not be stained by Giemsa. A possibility is that the halo was protein and RNA which had diffused out of the nucleus at lysis; a wider halo may be connected with the more efficient lysis by SDS than triton.

An argument against this theory is that a reproducible halo width would not be expected between experimental runs if the halo were simply due to the diffusion of small molecules out of the nucleus at lysis.

Attempts to stain with both EB and Giemsa were unsuccessful; this may indicate a finite number of attachment sites, the first stain blocking the attachment of the second.

7.7 Digestion of nucleoids with DNase

Digestion of nucleoids with DNase removed the outer halos of the triton-lysed nucleoids, leaving a central region with similar dimensions to the smaller centre diameters of intact nucleoids, and never as great as the larger centre diameters. Nuclease digestion of SDS-lysed nucleoids, however, left nothing visible remaining under bright field, fluorescence or SEM, agreeing with Cook (1984) who did not find a residual nuclear structure remaining after nuclease digestion of interphase nucleoids.

7.8 Assaying for DNA damage and repair

The dose-effect curves saturated at 4 Gy, somewhat lower than that seen in nucleoid sedimentation results; the dose-effect of Cook and Brazell (1975; 1976) was still increasing at 8 Gy, while the response of Van Rensburg *et al* (1985) plateaued by 5 Gy. Other nucleoid direct observation techniques where the cells are lysed in solution had not saturated by far higher doses than this; the direct observation dose-effect of Roti Roti and Wright (1987) had not plateaued by 20 Gy and Jaberaboansari *et al* (1988) did not see a plateau in their dose-effect by 25 Gy. The dose-response when using the microelectrophoresis technique did plateau at around 4 Gy (Ostling and Johanson, 1984), but cannot be interpreted in the same way as different parameters of the

nucleoid are being measured. It is possible that a related parameter is being measured.

A skewed distribution is expected when measured parameters are a cell-cycle-dependent distribution (Roti Roti and Wright, 1987). Even so, transformations are not generally carried out in direct measurement methods (Vogelstein *et al*, 1980; Roti Roti and Wright, 1987). Roti Roti and Wright (1987) showed that the distribution was no longer skewed when cells sorted from the G_1 or G_2 phase of the cell cycle only were used, so possibly more acceptable results would be obtained if a cell sorter could be used to select one of these populations for nucleoid production.

The repair kinetics are still not within small error limits but there does appear to be a general trend of a decrease in centre diameter with increasing repair time. A successful method of automation would allow more efficient scoring and would ultimately result in smaller uncertainties in the readings.

7.9 The effect of trypsin and electroporation on nucleoid structure

The results of the trypsin experiments also illustrate the effect of trypsin on cells and show the importance of the length of time of trypsinization to experimental results. The nucleoid technique investigated here was found to be sensitive to trypsinization time; this is an important fact to remember when using this technique in future. In fact it was not possible to quantitatively compare the effect of different lengths of time of trypsinization and methods of trypsinization, as the slides were not scorable. The electroporation experiments illustrate one of the limitations of the technique in that so much damage is done to the cells by just porating; with CHO cells, only 25% survive porating alone (Costa, unpublished results). Any cell

treatment is superimposed on top of this; the cell will therefore not behave as normal and must not be assumed to do so. Both trypsinization and electroporation are used to permeabilize cells but they produced nucleoids with very different morphologies. Electroporation resulted in altered bands crossing the entire nucleoid or total loss of halo, while trypsinization tended to make the halo irregular with pale sections, the centre rarely being altered. This could be indicating the different actions of the two methods; possibly trypsin mainly acts at the cell membrane while electroporation affects both the cell and nuclear membrane. Electroporation is known to mainly permeabilize the two poles of the cell perpendicular to the electric field, so possibly it is these regions which are indicated by the pale staining bands crossing the cells.

The electroporation results were unexpected however; electroporation alone perturbed the nucleoid structure to such an extent that the samples could not be scored. The dramatic irregular staining patterns had not previously been observed, especially the fact that they continued over both the centre and the halo regions. Possibly electroporation 'punches' holes in the cell and nuclear membrane, thus damaging both structures. Electroporation is known to damage cells, and if they are then subjected to a hypotonic shock one hour later, this may drastically alter the cellular structure, resulting in nucleoids with very abnormal appearances.

A 15 minute trypsinization additionally induced gross morphological changes in the DNA of most nucleoids in a population produced immediately after the formation of a cell suspension, indicating a major effect on many but not all cells; trypsinization therefore may not act equally on all cells in a population. At a late stage in the preparation of this thesis, the electroporation technique

was modified (Costa and Bryant, submitted for publication), whereby cells are porated then spun out of solution, resuspended in fresh medium and incubated for 8 hours. After this time, the medium was discarded along with two washes of trypsin and only the remaining cells are studied. This method is thought to discard the high proportion of cells which are heavily damaged by the poration procedure, but the remaining cells have still been successfully porated. Neutral elution experiments using the old and new methods gave the same dose-effect but the background elution level was almost eliminated using the new method.

7.10 Ideas on nucleoid structure

It is not possible to discuss the structures with any great certainty, but some ideas and suggestions will be discussed here. Investigations into nucleoid structure after lysing in triton and 1M NaCl reported in the literature (Cook and Brazell, 1975; 1976) show the dose response being expressed by changing diameter of the outer halo, the inner structure remaining constant at all doses and it is thought to be a residual nuclear lamina. In the technique developed here, setting cells in DW agarose before lysis, the dose effect was seen not by observing changes in the halo width but the diameter of the central region, which in addition was significantly larger than in the published techniques. The structure studied here is therefore not the same as that prepared for nucleoid sedimentation (Cook and Brazell, 1975) or the direct nucleoid observation techniques (Vogelstein *et al*, 1980). It was thought initially that the nucleoid centre was simply a residue of the nuclear lamina, a structure which is known to drastically change size after certain cellular perturbations including hypotonic treatment. The nuclear cage has been shown to be extremely flexible and will swell 2- to 5-fold after immersion in hypotonic solution unless the cage is

cross-linked (Jackson, 1986); a logical idea would therefore be that the large centre is due to the cage swelling due to the hypotonic treatment. However, immunoassay with antibodies to the 67kD lamin protein showed the lamina to be of a more constant diameter, approximating to the smallest nucleoid centre diameters seen and is probably the structure which was apparent in the centres of EB-stained nucleoids. Why the nucleoid centre changes diameter with dose is uncertain. It is possible that DNA is trapped within the hole left after cell lysis and when DNA breaks are introduced the molecular weight of the DNA is reduced and so can move out into the agarose slightly, thus increasing the observed central diameter. Observations made when attempting to automate the technique using the quantimet implied that the same total amount of material may be within the large and small centres and is just rather more diffuse and so covering a larger area in the large centres. This does not favour the alternative hypothesis that the centre expands because more material becomes trapped (possibly by cross-linking) within the centre, being prevented from moving out into the halo. This hypothesis of DNA being trapped within the hole in the agar would explain why the biphasic curve was seen for increasing EB concentrations; as EB intercalates, supercoiling is lost so the DNA attempts to stretch out. Diameters of cells when set in DW agarose just prior to lysis were measured to determine whether they were of an order of magnitude to fit in with this hypothesis. Cell diameters were $17^{+}/_{-}3$ μm, while nucleoid centre diameters were $24.7^{+}/_{-}0.3$ μm, which initially appears to refute this idea. Cell lysis was lengthy (90 minutes) to ensure that all cells would have lysed so the cells could possibly still be swelling when in lysis solution if it were possible that the agar could still be permanently deformed at this stage.

This idea does not explain why Giemsa staining should produce nucleoids with the same central diameter as the maximum EB central diameter; the peak in the Eth Br curve is thought to represent the removal of supercoiling by intercalation. Possibly Giemsa is such a large molecule that, even though it does not intercalate, when it binds to the DNA this also causes an expansion of the material within the hole in the agarose which again increases the diameter of the hole and so the observed central region. It is not certain why the DNA should expand to fill the hole in the agarose only when using DW agarose and not PBS agarose.

It is possible that DW gives a dose-effect as the continuous hypotonic/hypertonic state of the cells during nucleoid preparation inhibits repair. Hypotonic KCl is used to swell CHO nuclei before preparing metaphase aberrations and is known to induce the 2 chromatids to stay separated (Hughes, 1952). The hypotonic treatment could also be influencing the DNA here. The explanation that the mean centre diameter increases after X-irradiation as cells are killed and swell as they are degrading cannot hold, as this would not explain the response of the centre to Eth Br, or the repair kinetics observed, where the centres over-contract when repairing.

Evidence for the structure of the halo is therefore conflicting. The biphasic response with EthBr was seen in the centre diameter not the halo, indicating that the halo is not supercoiled DNA. Additionally, the halo after SDS treatment stained efficiently with Giemsa, which should not be seen if the halo were pure DNA with only traces of protein and implies significant quantities of protein are present. SDS halos also did not show a dose-effect, but varied significantly in width between experiments, possibly indicating different diffusion rates of the substance stained out of the centre in different experimental runs.

These observations indicate that the major proportion of the halo could be protein. However, Stainsall did not detect RNA or protein over any of the structure but only DNA, and DNase digestion removed the complete nucleoid structure except a small residual inner central region. Another possibility is that the halo is heavily damaged DNA as a result of the hypertonic shock before lysis which is known to induce the release of nucleases which will degrade DNA. This is unlikely, as the nucleases would have to totally fragment some areas of the chromatin, but leave other areas intact to produce the biphasic response to Eth Br. A likely possibility is that the halo consists of cellular proteins and RNA which diffused out of the cell at lysis and were then fixed into the agarosewhen it was dried as the DNase digestion was carried out before drying. DNase is not 100% specific and may contain contaminating RNase *etc* which may digest RNA in the halo structure. This idea could be verified by digesting the nucleoids with DNase after drying. The main evidence against the halo being protein is the blue Stainsall staining indicating the presence of DNA alone. Possibly the specificity of this batch of Stainsall is questionable and so this should be checked.

The halo of fibres seen when lysing nucleoids directly on the culture flask could be DNA and therefore would bear no relation to the putative RNA and protein halo seen when the cells were set in agarose before lysis.

There are clearly many questions left to be answered regarding the physical basis behind this technique. However, most techniques used to monitor DNA damage and repair are still not well understood; this causes major problems when attempts are made to interpret the results, for example, the controversy surrounding the lesion being monitored by neutral elution. The above, however, seemed the most reasonable explanations and models that could be formulated, based on the available

observations and data. It should be borne in mind that an *in vivo* system is not being studied here; cells have undergone major trauma during preparation and so results must be extrapolated back to the living cell situation with caution.

Chapter 7

GENERAL DISCUSSION

Chapter 7

The purpose of this work was to investigate the way in which trypsin potentiates X-ray damage and it subsequently also became necessary to determine whether the EDTA/buffer solution in which the trypsin was dissolved also contributed to the trypsin effect. The study was structured by considering various levels of damage; however these must be considered together to get an overall picture of how trypsin damages the cell.

Firstly, the effect of trypsin on X-ray cell killing was investigated. A small potentiation of X-ray damage was seen for CHO cells, verifying the existence of the previously reported trypsin effect. The trypsin effect in CHO cells was small compared with that previously seen in other cell lines. The reason for this difference was not known but could reflect differences between this and other cell lines in the sensitivity of the outer cell membrane to the enzyme. EAT (suspension) cells were also assayed; the trypsin effect had not been studied before in this line. It was concluded that a change in cellular morphology may be an important factor in trypsin-induced cell killing, as has been suggested by Reddy *et al*, (1989), as trypsin treatment had no effect on EAT cell killing. Possibly it is the removal of cell 'feet' to detach them from the surface which leaves 'holes' in the cell surface and which in turn causes the cell to be more permeable; this is not thought to occur in cells trypsinized in suspension (Lamb and Ogden, 1987).

Attempts to clarify these effects at the chromosome and DNA level and so gain information on the mechanism of the trypsin effect led to some interesting but conflicting results.

Firstly, the lack of potentiation of EAT cell killing did not correlate with the clear trypsin effect on the frequency of X-ray induced chromosome aberrations. Trypsin treatment increased the frequency of X-ray induced chromosomal aberrations (CA) in EAT cells over those buffer (EDTA) treated. This was due to an increase in frequency of fragments but not bridges which was thought to imply that trypsin potentiates X-ray cell killing by inhibiting repair rather than by increasing the rate of mis-repair. This therefore makes the lack of trypsin effect on cell killing in EAT cells seem paradoxical and does not support the theory of Joshi *et al* (1982) who proposed that the loss of chromosome fragments after the first mitosis was connected with cell killing. The plating efficiency of EAT cells was not high (69%) so a possible explanation for the apparent paradox might be that the increase in aberration frequency after trypsin treatment and irradiation was associated with the 30% of the EAT cell population for which reproductive integrity is not maintained even in the control samples; those cells which are not viable could also be less capable of DNA repair and so would accumulate chromosomal fragments. This theory was discounted however by analysis of the CA data which showed that trypsin acts by increasing the number of cells which contain CA rather than by increasing the frequency of aberrations in those cells which already have CA. No explanation could be reached for this discrepancy.

Secondly, the results obtained with the DNA unwinding and neutral elution methods indicate effects of both trypsin and EDTA on the induction and repair of damage. The concept of EDTA also potentiating cell killing had not previously been reported. Both trypsin and EDTA increased the frequency of induction of dsb as measured by elution, although it could be that this is an effect on the ability of damaged DNA to elute through filters rather than an actual increase in breakage

of DNA, as results obtained with the unwinding method indicated no difference in initially induced damage. These findings are important, because regardless of the cause, if routine trypsinization affects the dose-effect relationship with neutral elution, this could affect experiments where cells are trypsinized at varying times before irradiation and elution. They also show that EDTA alone may make a major contribution to the potentiation of X-ray damage; trypsin treated cells (in EDTA/buffer) did not show a greater rate of induction of dsb than those treated with EDTA/buffer alone.

Bender *et al* (1974) proposed that CA arose from dsb which are not repaired. These results do not support this theory because an increase in anaphase CA after trypsinization compared with buffer treated cells did not correspond with a similar effect on the dsb repair capacity as observed using DNA unwinding and neutral elution. No difference between the DNA dsb repair of trypsin and buffer treated cells was seen, but these both inhibited repair over the untreated samples.

In contrast, repair of DNA ssb was clearly affected by trypsin, and EDTA did not affect this repair when monitored by DNA unwinding although these ssb effects should not influence cell kill.

The DNA unwinding and neutral elution results imply that the trypsin effect could occur possibly by trypsin and/or EDTA increasing the frequency of induction of dsb and also by trypsin and EDTA inhibiting dsb repair. This also implies that, contrary to the hypothesis of Phillips and Tolmach (1964) and Berry *et al* (1966), cell cycle effects such as synchronization and accumulation in resistant cell cycle phases cannot be solely responsible for the trypsin effect, as potentiation was seen in cells X-irradiated soon after trypsinization.

Reddy *et al* (1989) noted no difference in morphology when mitotic cells were trypsinized but only used light microscopy. It would be

interesting to carry out an SEM comparison of trypsinized cells, mitotic cells and trypsinized mitotic cells to see if any differences were evident.

Although trypsin was shown to have an effect on chromosomal abnormalities and DNA repair in irradiated cells, and the nucleoid experiments also imply that the chromatin is affected in some way by trypsin, no direct evidence was found to suggest that trypsin is still active after it is taken up into the cell, enabling molecules to interact directly with the DNA.

No difference in the unwinding kinetics of trypsinized and EDTA treated cells was seen, implying no major difference in conformation due to these treatments. This would have been expected if trypsin treatment was causing relaxation or contraction of the chromosomes as suggested by Reddy *et al*, (1989). It is possible however that both these treatments affected the unwinding kinetics to the same extent, the untreated kinetics being different. No extra effect of trypsin over EDTA was seen on cell killing or dsb repair, implying that trypsin has no additional effect on these assays, possibly because the potentiating effect of trypsin and EDTA on X-ray damage is a saturable process. Lamb and Ogden (1987) saw an increase in permeability after obtaining a cell suspension with EDTA only as well as when using trypsin alone, so possibly the EDTA/buffer used here does also play a role in the trypsin effect. However, Lamb and Ogden (1987) found that neither trypsin nor EDTA treatment of suspension cultured cells increased cellular permeability, as opposed to those attached.

A new nucleoid assay was developed which showed that trypsin was affecting the nucleoid structure, but the way in which it occurred meant that the alterations could be observed but not quantitated. The technique provided some interesting information on nucleoid structure

but is was difficult to make conclusions from the observations as the structure being observed was not fully elucidated.

As EDTA alone also inhibited repair of dsb but not of ssb, then this may be due to EDTA chelating ions which are necessary to the enzymes involved in dsb repair if EDTA entered the cell in an active form.

Another explanation is that simply by making the cells more permeable trypsin disrupts the ionic concentration within the cell, the resulting loss of low molecular weight components reducing cellular repair capacity and the entry of trypsin into the cell being purely incidental to the trypsin effect. X-rays do damage all cell components at random, not just the DNA, including the cell membrane - possibly the trypsin interacts with this damage to make it worse or fix the damage. A 15 minute trypsinization induced gross morphological changes in the DNA of most nucleoids in a population produced immediately after the formation of a cell suspension, indicating a major effect on many but not all cells, so trypsin may not act equally on all cells in a population.

The observed effects could also be caused indirectly by damage to the cell membrane leading to alterations in ionic balance or loss of vital molecules. The trypsin effect cannot therefore be directly connected with cell attachment (flattening) and detachment (rounding) as has been suggested (Reddy *et al*, 1989), and as the EAT survival curve results in isolation implied, as trypsin clearly potentiated the frequency of X-ray induced CA in EAT cells which grow in suspension. It was the detachment of cell 'feet' as part of the cell suspension process which was thought to make holes in the cell, thereby making them more permeable, unbalanced and so less capable of repair.

The work could be continued in various directions. The nucleoid technique developed could be extremely useful if the problems with automation could be overcome and it would be interesting to investigate the effect of restriction endonucleases on cells using alternative methods of permeabilization which would not damage the cells to such an extent. A useful experiment would be to repeat the EAT survival curve but with attached EAT grown in MEM to determine whether or not the potentiation effect was still negligible. Other cell lines could also be tried such as WeHi, which attach loosely so can either be trypsinized or shaken off the surface. Additionally, it would be interesting to investigate the effect of trypsin on cells in different phases of the cell cycle, and also to alter the trypsinization procedure itself, such as the length of time, temperature, and trypsin concentration used. The same experiments reported here could also be carried out using other cell detachment methods as discussed in Chapter 1.

There is little to offer by way of explanation of these reported effects. However in view of the ubiquity of the trypsin/EDTA procedures in radiation biology, it is important that they be considered as the cause of potential variability in experiments. The reason for carrying out this work was the fact that large proteins (restriction endonucleases) will enter trypsinized cells and reach the nuclear DNA (Bryant, 1984, 1988). Trypsin (and EDTA) may therefore be similarly able to enter cells and cause damage to intracellular structures including DNA associated proteins. The results with unwinding and neutral elution may support this theory. It is clear from the results of these experiments that the length of time of trypsinization has a critical influence on the severity of the trypsin effect so it is essential to trypsinize for the same (minimum) length of time in all

experiments to be compared, and also at the same time interval before irradiation.

In conclusion, the trypsin effect has been verified as previously reported and in addition shown that effects of trypsin on chromosomal damage in X-irradiated cells may be even more marked than those at the cellular level. Possibly linked to the cellular and chromosomal effects are the effects of trypsin and EDTA on the induction of damage and particularly on the reduced rate of repair of both ssb and dsb.

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APPENDIX I

Table 6.1 Mean nucleoid measurements from a range of lysis methods

AGAR SOL[N]	LYSIS DETERG[T]	LYSIS SALT CONC[N]	X-RAY DOSE	CENTRE	TOTAL DIAM[R]	HALO
DIST[D] WATER	TRITON pH7.4	0M	0Gy	-	15.336	-
			3Gy	-	15.120	-
		0.15M	0Gy	24.030	26.142	1.056
			3Gy	23.850	25.518	0.834
		1M	0Gy1	25.134	41.922	8.394
			0Gy2	25.062	40.830	7.884
			3Gy1	26.652	40.122	6.735
			3Gy2	26.754	41.232	7.239
		2M	0Gy1	22.254	44.226	10.986
			0Gy2	24.888	42.978	9.045
			3Gy1	24.108	44.328	10.110
			3Gy2	24.558	45.180	10.311
PBS	TRITON pH7.4	0M	0Gy	-	16.632	-
			3Gy	-	16.776	-
		0.15M	0Gy	14.754	24.864	5.055
			3Gy	15.354	25.518	5.082
		1M	0Gy1	14.862	45.492	15.315
			0Gy2	14.460	48.204	16.872
			3Gy1	14.898	48.348	16.725
			3Gy2	14.634	47.034	16.200
		2M	0Gy1	14.502	45.060	15.279
			0Gy2	14.322	48.408	17.043
			3Gy1	14.274	46.554	16.140
			3Gy2	14.310	44.172	14.931
PBS	SDS pH7.4	0M	0Gy1	15.144	57.516	21.186
			0Gy2	15.768	54.318	19.275
			3Gy1	15.306	54.240	19.467
			3Gy2	15.018	58.548	21.765
PBS	SDS pH9.5	0M	0Gy1	15.444	61.566	23.061
			0Gy1	16.308	58.632	21.162
			3Gy1	15.228	53.862	19.317
			3Gy2	15.588	55.944	20.178

Table 6.2 – Probability plot correlation coefficients using actual measurements (um).

AGAR SOLN	LYSIS DETERGT	LYSIS SALT CONCN	X-RAY DOSE	CENTRE	TOTAL DIAMR	HALO
DISTD WATER	TRITON pH7.4	0M	0Gy	–	0.940	–
			3Gy	–	0.962	–
		0.15M	0Gy	0.985	0.985	**0.990**
			3Gy	0.985	0.984	**0.996**
		1M	0Gy1	0.978	**0.993**	0.980
			0Gy2	0.986	**0.987**	**0.989**
			3Gy1	0.979	0.983	0.964
			3Gy2	0.963	0.980	0.982
		2M	0Gy1	**0.989**	0.985	0.970
			0Gy2	**0.987**	**0.987**	0.971
			3Gy1	0.972	0.981	**0.992**
			3Gy2	0.978	**0.993**	0.981
PBS	TRITON pH7.4	0M	0Gy	–	0.944	–
			3Gy	–	0.927	–
		0.15M	0Gy	0.980	0.969	0.983
			3Gy	0.935	0.982	0.980
		1M	0Gy1	0.977	0.977	**0.988**
			0Gy2	0.951	**0.988**	**0.987**
			3Gy1	0.960	0.958	**0.988**
			3Gy2	0.927	0.953	0.972
		2M	0Gy1	**0.988**	0.983	**0.996**
			0Gy2	0.984	**0.990**	**0.991**
			3Gy1	**0.994**	0.970	**0.993**
			3Gy2	0.972	0.962	**0.989**
PBS	SDS pH7.4	0M	0Gy1	0.915	0.961	**0.991**
	pH9.5	0M	0Gy1	0.863	0.944	0.985
			0Gy2	0.885	0.935	0.936
			3Gy1	0.977	**0.989**	**0.995**
			3Gy2	0.915	0.940	0.960

Table 6.3 - Probability plot correlation coefficients using the square roots of nucleoid measurements.

AGAR SOL^{N}	LYSIS DETERG^{T}	LYSIS SALT CONC^{N}	X-RAY DOSE	CENTRE	TOTAL DIAM^{R}	HALO
DIST^{D} WATER	TRITON pH7.4	0M	0Gy	-	0.957	-
			3Gy	-	0.974	-
		0.15M	0Gy	**0.993**	**0.992**	0.976
			3Gy	**0.991**	**0.991**	**0.983**
		1M	0Gy1	**0.989**	**0.990**	**0.985**
			0Gy2	**0.993**	**0.993**	**0.995**
			3Gy1	**0.987**	**0.988**	0.980
			3Gy2	0.972	**0.985**	**0.988**
		2M	0Gy1	**0.990**	**0.990**	0.965
			0Gy2	**0.983**	**0.986**	**0.991**
			3Gy1	**0.990**	**0.990**	0.971
			3Gy2	**0.986**	**0.996**	0.977
PBS	TRITON pH7.4	0M	0Gy	-	0.959	-
			3Gy	-	0.946	-
		0.15M	0Gy	**0.987**	0.978	**0.992**
			3Gy	0.956	**0.988**	0.903
		1M	0Gy1	**0.985**	**0.983**	**0.994**
			0Gy2	0.966	**0.991**	**0.985**
			3Gy1	0.974	0.965	**0.992**
			3Gy2	0.945	0.959	0.979
		2M	0Gy1	**0.994**	**0.986**	**0.995**
			0Gy2	**0.990**	**0.993**	**0.989**
			3Gy1	**0.997**	0.972	**0.994**
			3Gy2	**0.982**	0.969	**0.993**
PBS	SDS pH7.4	0M	0Gy1	0.939	0.969	**0.994**
			0Gy2	**0.987**	**0.995**	**0.997**
			3Gy1	0.979	0.965	0.981
			3Gy2	0.971	0.970	**0.986**
	SDS pH9.5	0M	0Gy1	0.897	0.958	**0.991**
			0Gy2	0.918	0.948	0.954
			3Gy1	**0.985**	**0.992**	**0.994**
			3Gy2	0.939	0.950	0.971

Table 6.4 Probability plot correlation coefficients using $\log_e$ of nucleoid measurements

AGAR SOL[N]	LYSIS DETERG[T]	LYSIS SALT CONC[N]	X-RAY DOSE	CENTRE	TOTAL DIAM[R]	HALO
DIST[D] WATER	TRITON pH7.4	0M	0Gy	-	0.970	-
			3Gy	-	0.984	-
		0.15M	0Gy	0.997	0.997	0.993
			3Gy	0.994	0.996	0.987
		1M	0Gy1	0.994	0.986	0.982
			0Gy2	0.994	0.996	0.989
			3Gy1	0.992	0.991	0.989
			3Gy2	0.980	0.990	0.989
		2M	0Gy1	0.985	0.993	0.956
			0Gy2	0.991	0.990	0.981
			3Gy1	0.989	0.992	0.966
			3Gy2	0.992	0.997	0.965
PBS	TRITON pH7.4	0M	0Gy	-	0.971	-
			3Gy	-	0.961	-
		0.15M	0Gy	0.990	0.986	0.987
			3Gy	0.973	0.993	0.929
		1M	0Gy1	0.990	0.987	0.997
			0Gy2	0.977	0.993	0.979
			3Gy1	0.984	0.971	0.993
			3Gy2	0.961	0.965	0.984
		2M	0Gy1	0.997	0.989	0.994
			0Gy2	0.992	0.995	0.985
			3Gy1	0.998	0.975	0.996
			3Gy2	0.989	0.974	0.996
PBS	SDS pH7.4	0M	0Gy1	0.958	0.975	0.996
			0Gy2	0.992	0.995	0.996
			3Gy1	0.986	0.974	0.987
			3Gy2	0.980	0.975	0.990
PBS	SDS pH9.5	0M	0Gy1	0.926	0.969	0.994
			0Gy2	0.944	0.959	0.968
			3Gy1	0.991	0.992	0.990
			3Gy2	0.959	0.959	0.980

Table 6.5 Probability plot correlation coefficients using $-1/x$ of nucleoid measurements

AGAR SOL[N]	LYSIS DETERG[T]	LYSIS SALT CONC[N]	X-RAY DOSE	CENTRE	TOTAL DIAM[R]	HALO
DIST[D] WATER	TRITON pH7.4	0M	0Gy	-	0.970	-
			3Gy	-	0.984	-
		0.15M	0Gy	0.993	0.997	0.803
			3Gy	0.993	0.998	0.927
		1M	0Gy1	0.990	0.973	0.926
			0Gy2	0.983	0.998	0.924
			3Gy1	0.994	0.995	0.990
			3Gy2	0.988	0.996	0.980
		2M	0Gy1	0.961	0.995	0.924
			0Gy2	0.997	0.995	0.925
			3Gy1	0.980	0.991	0.929
			3Gy2	0.994	0.998	0.923
PBS	TRITON pH7.4	0M	0Gy	-	0.971	-
			3Gy	-	0.961	-
		0.15M	0Gy	0.986	0.995	0.928
			3Gy	0.991	0.997	0.774
		1M	0Gy1	0.996	0.994	0.997
			0Gy2	0.991	0.994	0.954
			3Gy1	0.996	0.982	0.994
			3Gy2	0.983	0.975	0.987
		2M	0Gy1	0.999	0.993	0.986
			0Gy2	0.984	0.997	0.970
			3Gy1	0.992	0.979	0.994
			3Gy2	0.995	0.984	0.995
PBS	SDS pH7.4	0M	0Gy1	0.984	0.986	0.996
			0Gy2	0.998	0.994	0.989
			3Gy1	0.988	0.987	0.955
			3Gy2	0.991	0.982	0.994
PBS	SDS pH9.5	0M	0Gy1	0.966	0.984	0.995
			0Gy2	0.979	0.976	0.986
			3Gy1	0.995	0.990	0.976
			3Gy2	0.982	0.973	0.991

Table 6.7

Analysis of Variance of Various Lysis Treatments

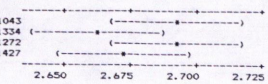
Table giving mean, standard deviation and individual 95 per cent confidence intervals for mean based on pooled standard deviation of each sample's ln transformed centre, total diameter and halo measurements.

Triton PB 1M

1. Centre

Pooled SD 0.1277

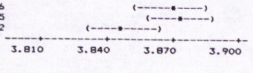
Dose	N	Mean	SD
0Gy 1	100	2.6933	0.1043
0Gy 2	100	2.6622	0.1334
3Gy 1	100	2.6929	0.1272
3Gy 2	100	2.6727	0.1427



2. Total Diameter

Pooled SD 0.0823

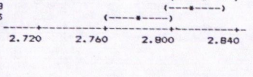
Dose	N	Mean	StDev
0Gy1	100	3.8128	0.0967
0Gy2	100	3.8726	0.0756
3Gy1	100	3.8758	0.0725
3Gy2	100	3.8474	0.0822



3. Halo Width

Pooled SD 0.0998

Dose	N	Mean	StDev
0Gy1	100	2.7214	0.1224
0Gy2	100	2.8206	0.1017
3Gy1	100	2.8137	0.0808
3Gy2	100	2.7810	0.0893

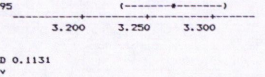


Triton DW 1M

1. Centre

Pooled SD 0.2098

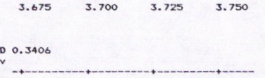
Dose	N	Mean	StDev
0Gy1	100	3.2104	0.2132
0Gy2	100	3.1926	0.2417
3Gy1	100	3.2628	0.1997
3Gy2	100	3.2703	0.1795



2. Total Diameter

Pooled SD 0.1131

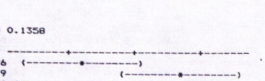
Dose	N	Mean	StDev
0Gy1	100	3.7288	0.1205
0Gy2	100	3.7020	0.1218
3Gy1	100	3.6863	0.1060
3Gy2	100	3.7139	0.1030



3. Halo

Pooled SD 0.3406

Dose	N	Mean	StDev
0Gy1	100	2.0667	0.3600
0Gy2	100	1.9886	0.4050
3Gy1	100	1.8648	0.2894
3Gy2	100	1.9369	0.2946

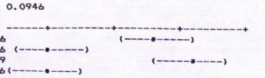


SDS pH 7.4

1. Centre

Pooled SD 0.1358

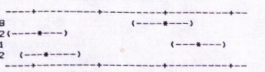
Dose	N	Mean	StDev
0Gy1	100	2.7053	0.1526
0Gy2	100	2.7513	0.1149
3Gy1	100	2.7016	0.1224
3Gy2	100	2.7169	0.1495



2. Total Diameter

Pooled SD 0.0946

Dose	N	Mean	StDev
0Gy1	100	4.0468	0.1016
0Gy2	100	3.9913	0.0846
3Gy1	100	4.0653	0.0949
3Gy2	100	3.9887	0.0966



3. Halo Width

Pooled SD 0.1079

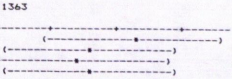
Dose	N	Mean	StDev
0Gy1	100	3.081	0.1028
0Gy2	100	2.9535	0.1042
3Gy1	100	3.0742	0.1101
3Gy2	100	2.9621	0.1142

Triton PB 2M

1. Centre

Pooled SD 0.1363

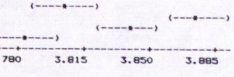
Dose	N	Mean	StDev
0Gy1	100	2.6653	0.1343
0Gy2	100	2.6519	0.1410
3Gy1	100	2.6488	0.1394
3Gy1	100	2.6524	0.1302



2. Total Diameter

Pooled SD 0.0825

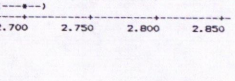
Dose	N	Mean	StDev
0Gy1	100	3.8044	0.0851
0Gy2	100	3.8762	0.0837
3Gy1	100	3.8383	0.0679
3Gy2	100	3.7839	0.0915



3. Halo Width

Pooled SD 0.0974

Dose	N	Mean	StDev
0Gy1	100	2.7221	0.0948
0Gy2	100	2.8298	0.1107
3Gy1	100	2.7786	0.0745
3Gy2	100	2.6979	0.1057

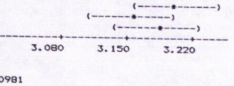


Triton DW 2M

1. Centre

Pooled SD 0.235

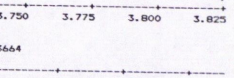
Dose	N	Mean	StDev
0Gy1	100	3.0556	0.3148
0Gy2	100	3.1997	1.1701
3Gy1	100	3.1559	0.2334
3Gy2	100	3.1818	0.1956



2. Total Diameter

Pooled SD 0.0981

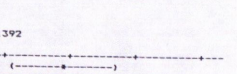
Dose	N	Mean	StDev
0Gy1	100	3.7842	0.1012
0Gy2	100	3.7565	0.0909
3Gy1	100	3.7861	0.1051
3Gy2	100	3.8062	0.0944



3. Halo Width

Pooled SD 0.3664

Dose	N	Mean	StDev
0Gy1	100	2.3189	0.4154
0Gy2	100	2.1650	0.2830
3Gy1	100	2.2333	0.4196
3Gy2	100	2.2837	0.3293

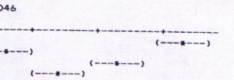


SDS pH 9.5

1. Centre

Pooled SD 0.1392

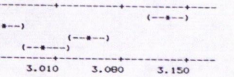
Dose	N	Mean	StDev
0Gy1	100	2.7273	0.1354
0Gy2	100	2.7152	0.1256
3Gy1	100	2.7786	0.1554
3Gy2	100	2.7364	0.1388



2. Total Diameter

Pooled SD 0.1046

Dose	N	Mean	StDev
0Gy1	100	4.1136	0.1130
0Gy2	100	3.9818	0.0964
3Gy1	100	4.0650	0.1130
3Gy2	100	4.0195	0.0976

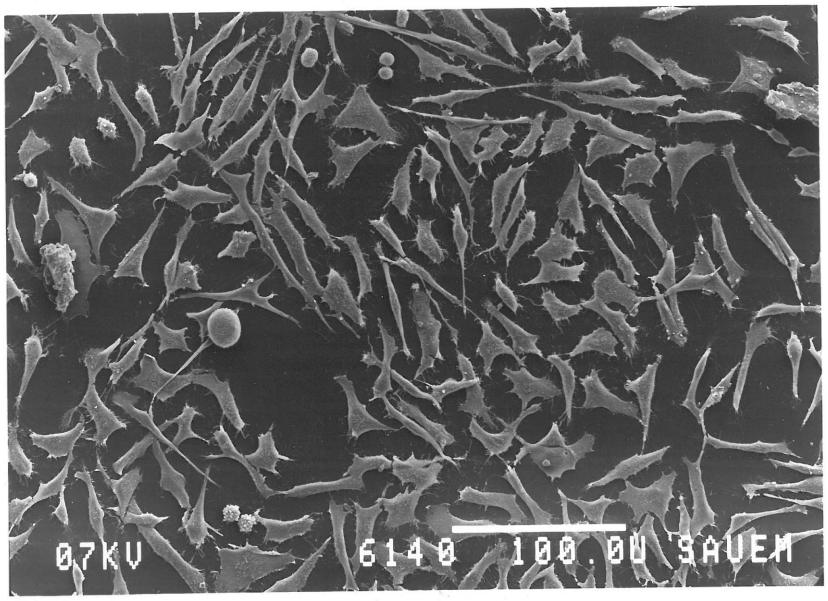


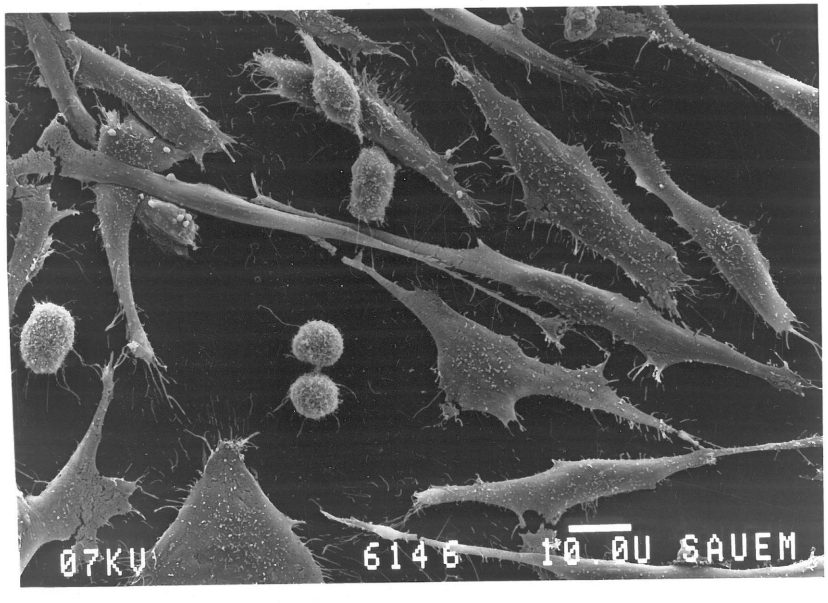
3. Halo Width

Pooled SD 0.1193

Dose	N	Mean	StDev
0Gy1	100	3.1299	0.1282
0Gy2	100	2.9540	0.1198
3Gy1	100	3.0448	0.1202
3Gy2	100	2.9986	0.1084

APPENDIX II


07KV
6140 100.0U SAUEM


07KV
6146 10.0U SAUEM

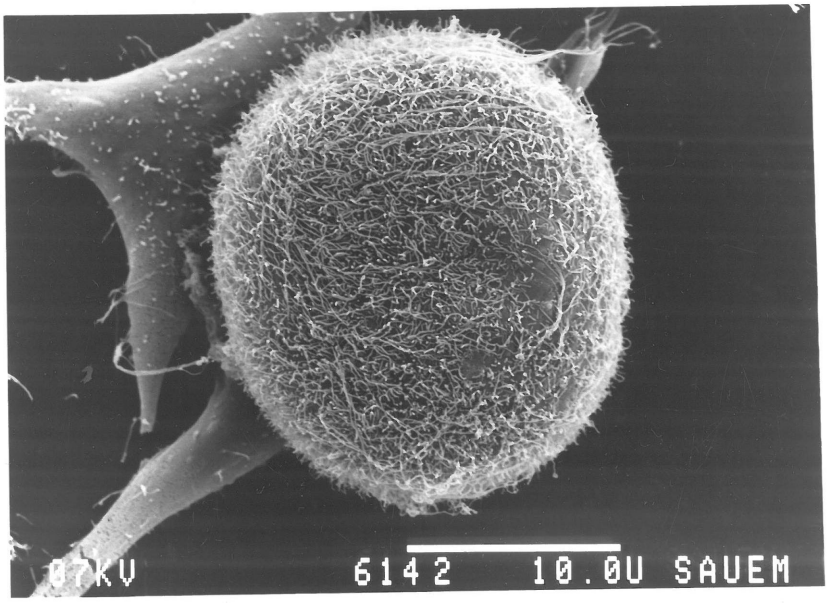

07KV
6142 10.0U SAUEM

Plate 1

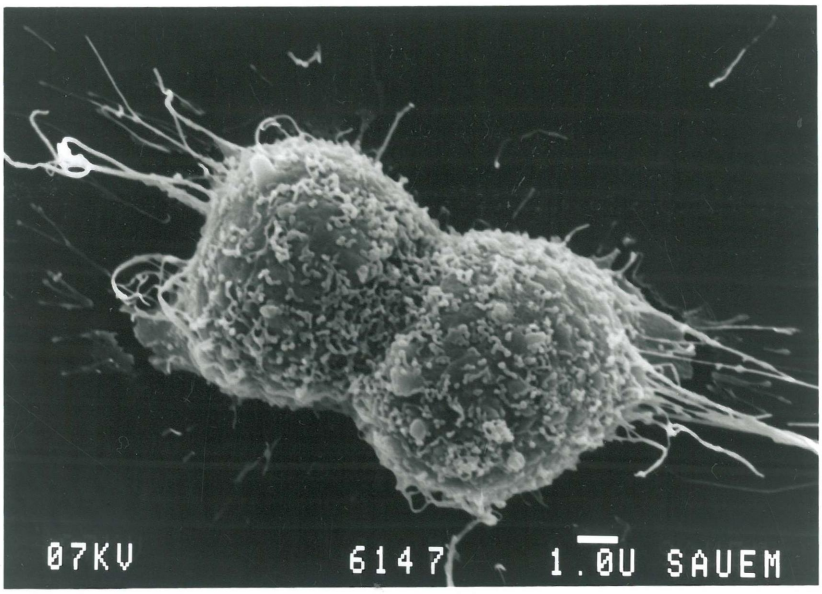
Scanning electron micrographs of asynchronous CHO cells growing attached to the culture flask surface.

Bars give scale in microns.

(a) Low magnification field

(b) Higher magnification field

(c) Tetraploid cell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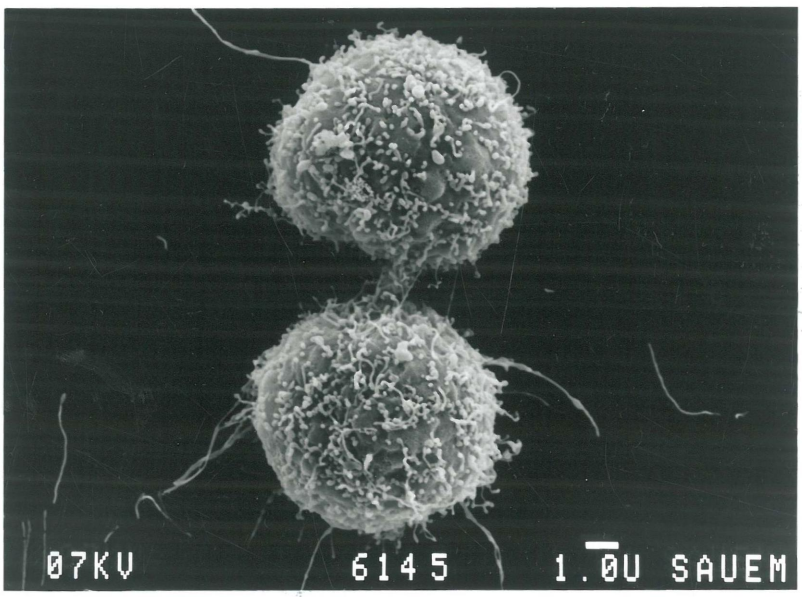
6147
1.0U SAUEM


07KV
6145
1.0U SAUEM

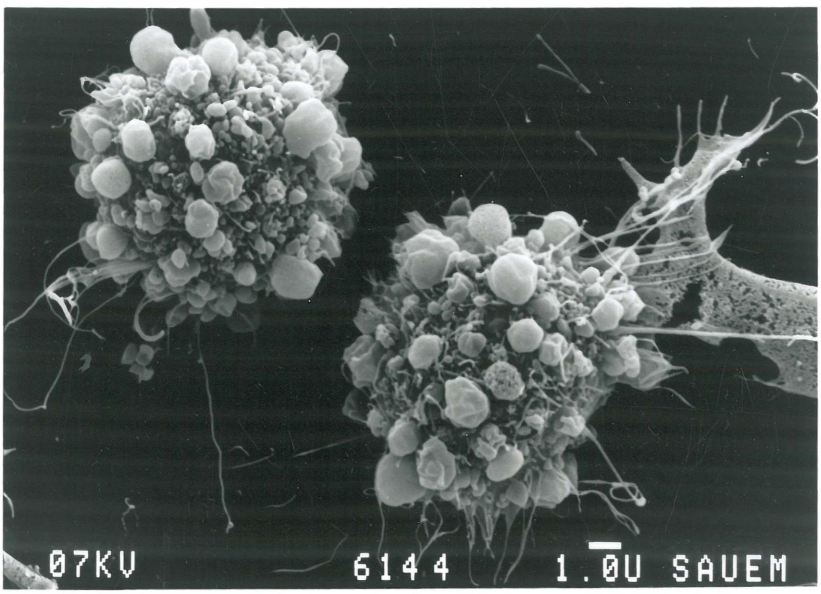

07KV
6144
1.0U SAUEM

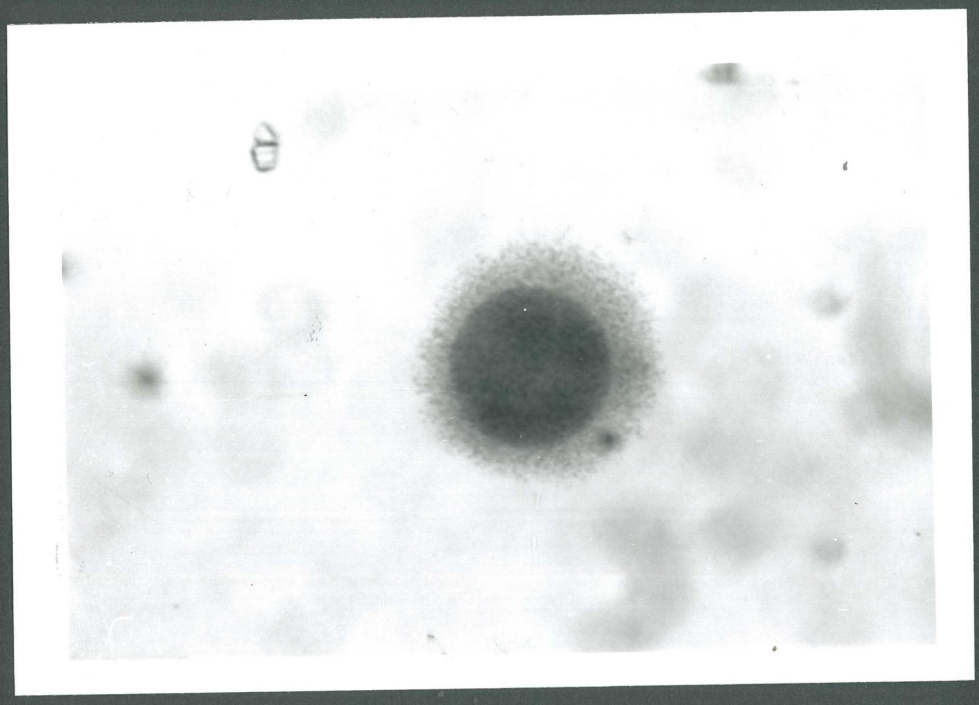
Plate 2

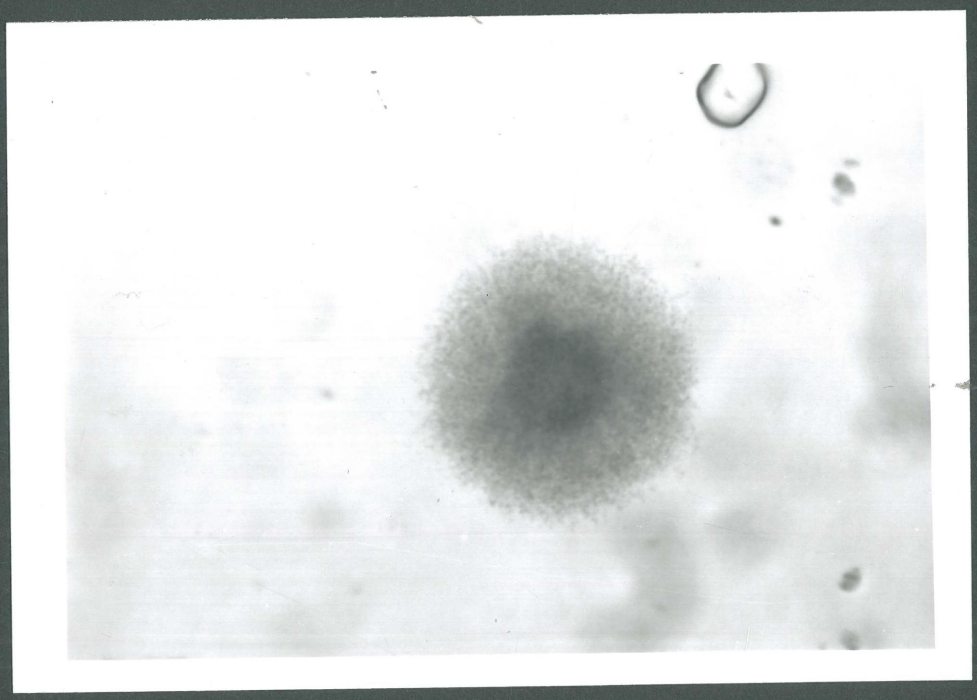
Scanning electron micrographs of CHO cells undergoing division. Bars give scale in microns

(a)

(b)

(c)





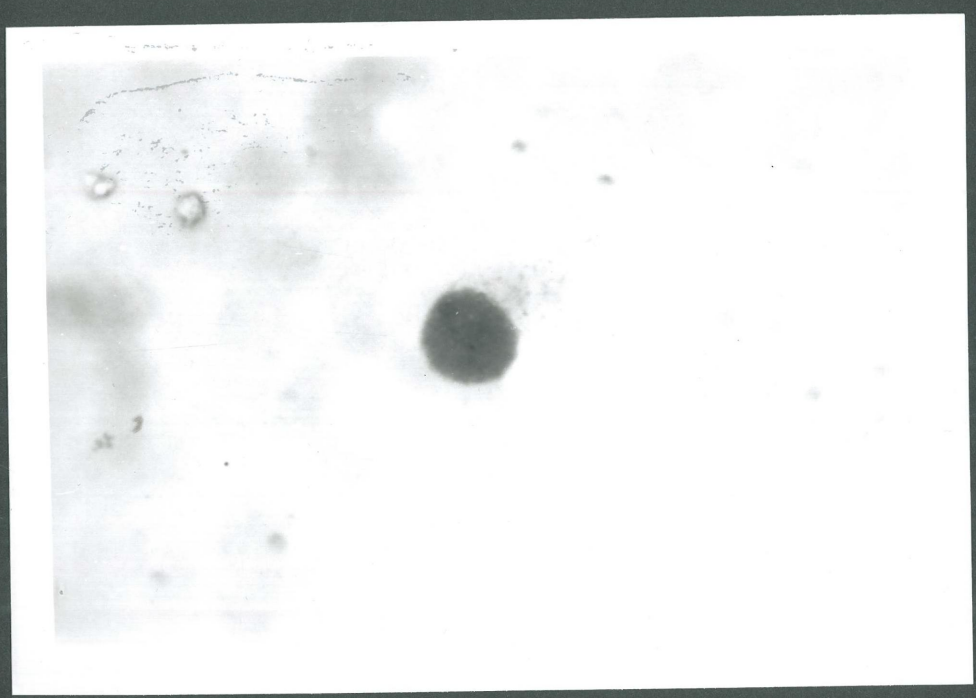


Plate 3

Micrographs of nucleoids set in DW agarose, lysed in triton and stained with Giemsa. (a) and (b) illustrate the range of morphologies seen.

(a) Lysed in 1M NaCl

Centre diameter 26.4 μm

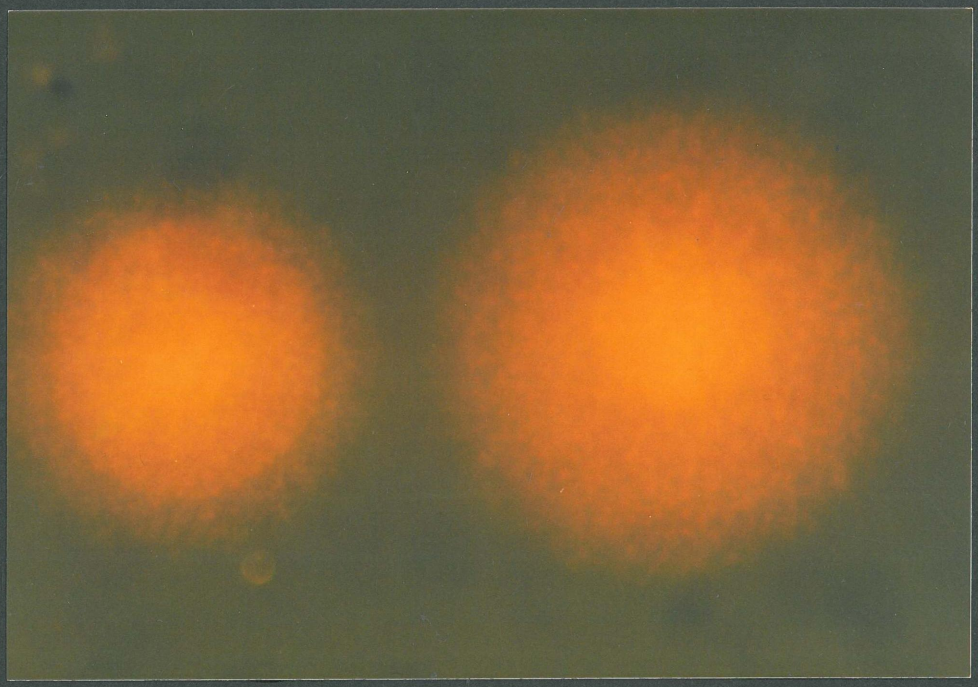
(b) Lysed in 1M NaCl

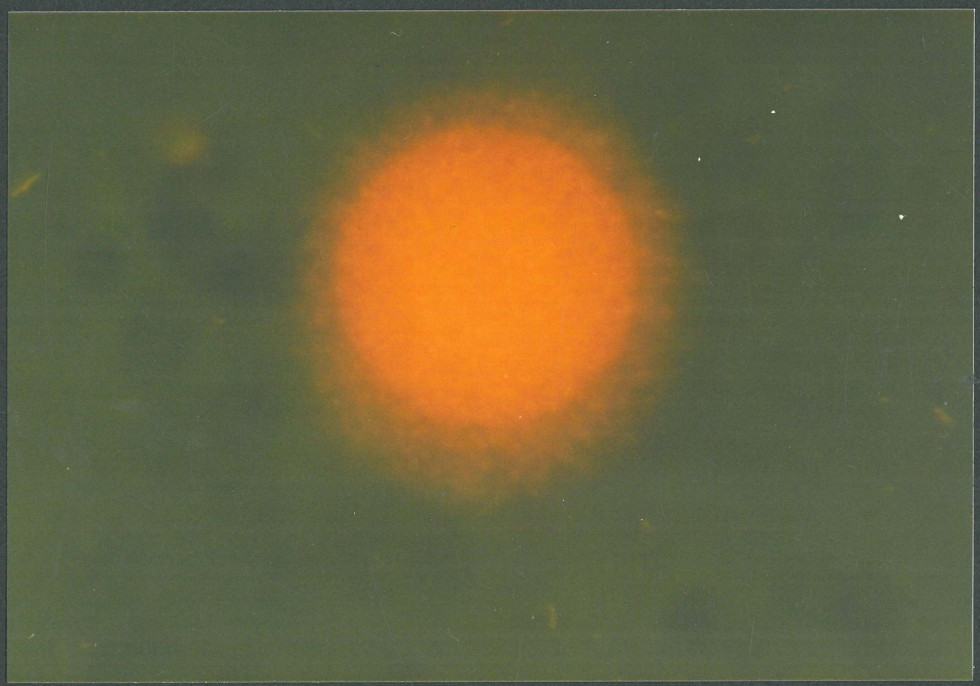
Centre diameter 17.7 μm

(c) Lysed in 0M NaCl

Centre diameter 15.6 μm

To compare the centre size of the small-centred 1M NaCl lysed nucleoid and the 0M NaCl nucleoids





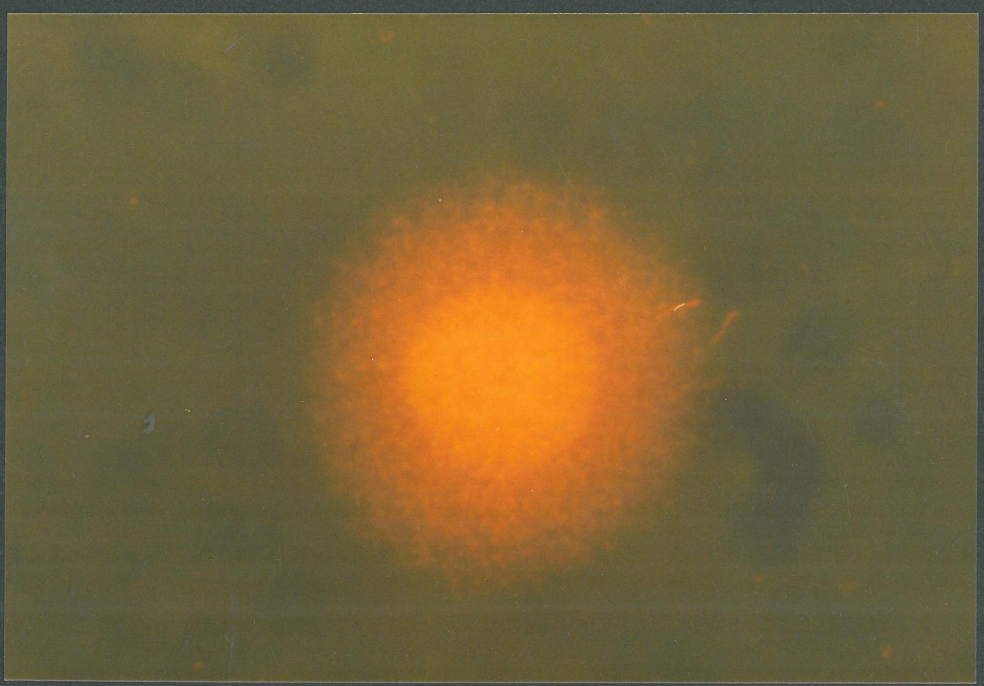


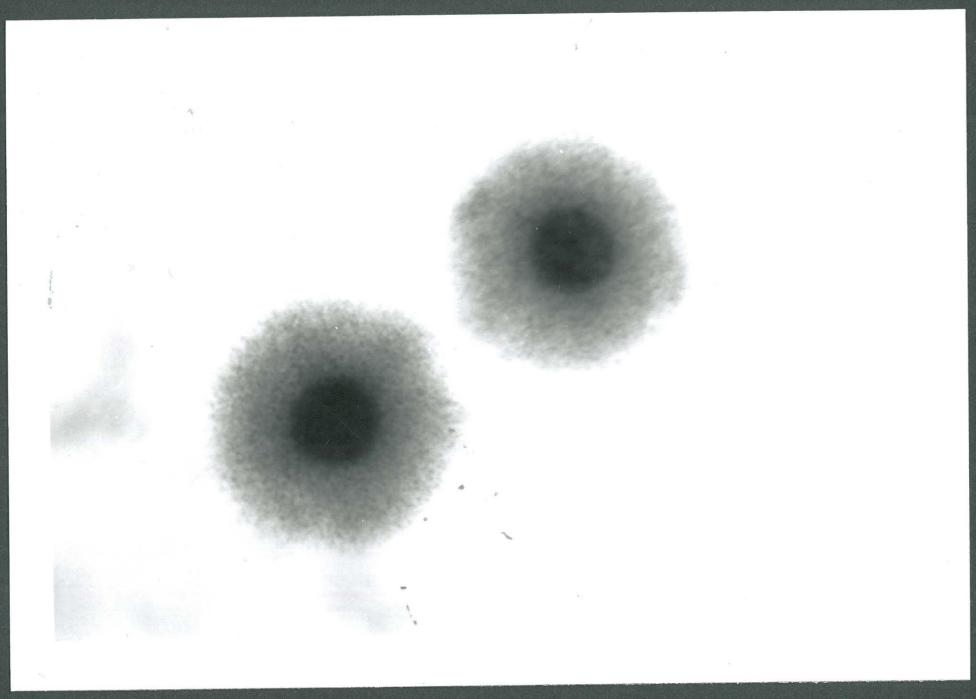
Plate 4

Micrographs of nucleoids of cells set in DW agarose, lysed in triton and 1M NaCl and stained with ethidium bromide. The range of morphologies seen are illustrated.

(a) Centre diameter 25.9 μm and 17.05 μm

(b) Centre diameter 27.4 μm

(c) Centre diameter 17.73 μm



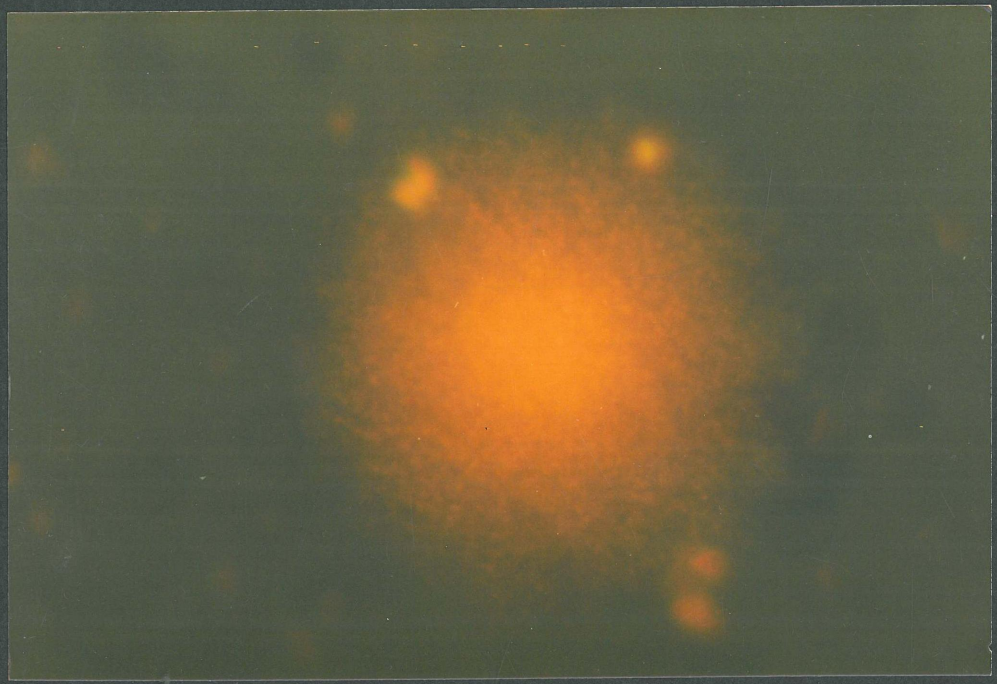


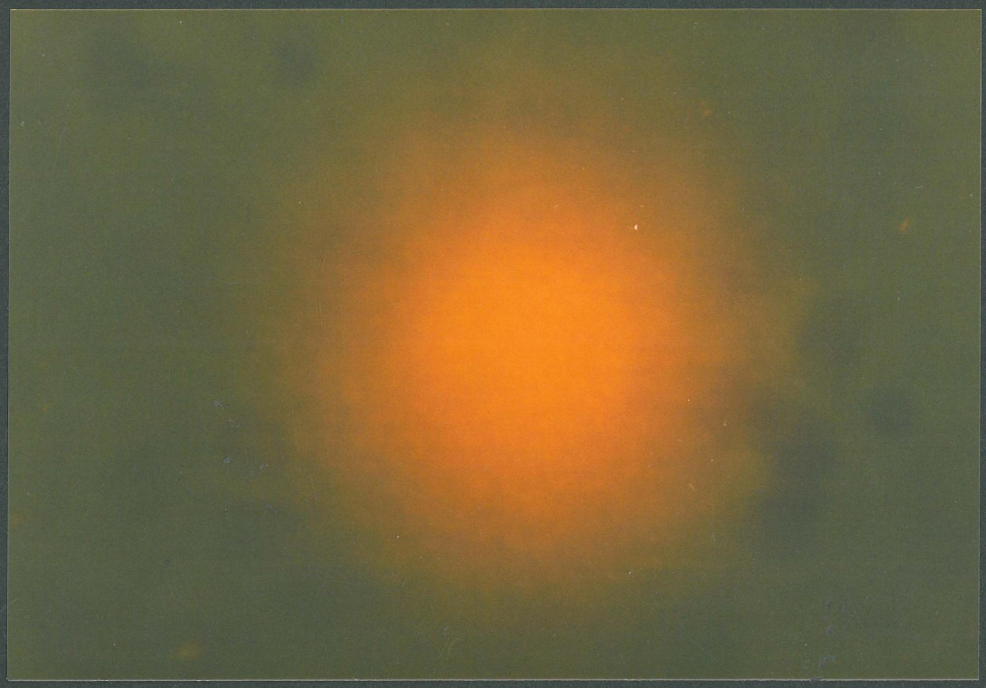
Plate 5

Micrographs of nucleoids of cells set in PBS agarose and lysed in triton with 1M NaCl.

(a) stained with Giemsa

To illustrate the uniformity of nucleoid size.

(b) Stained with ethidium bromide



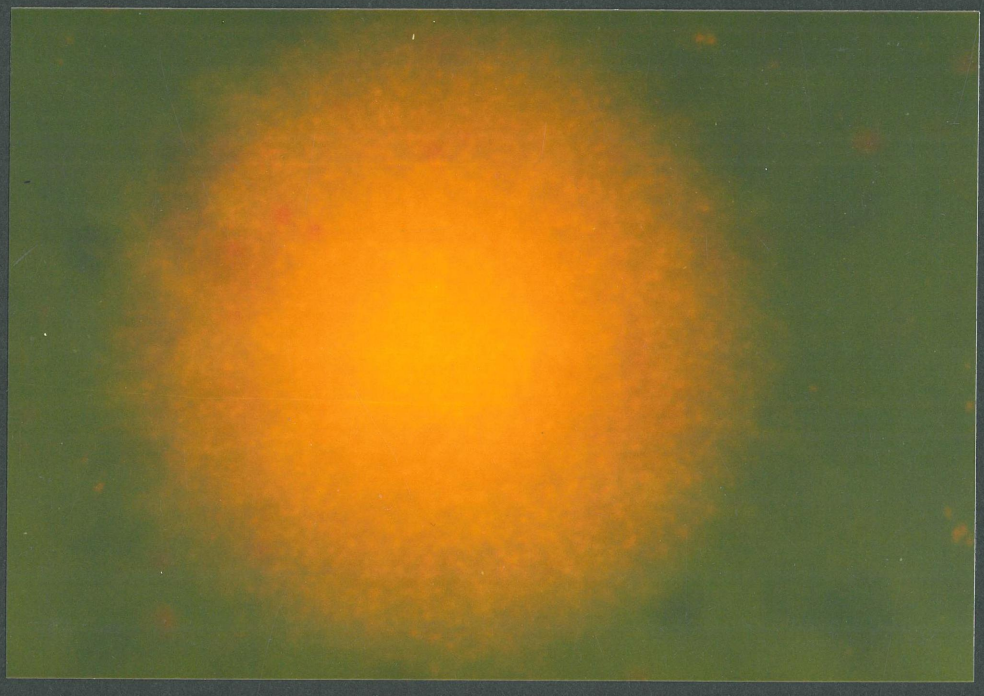
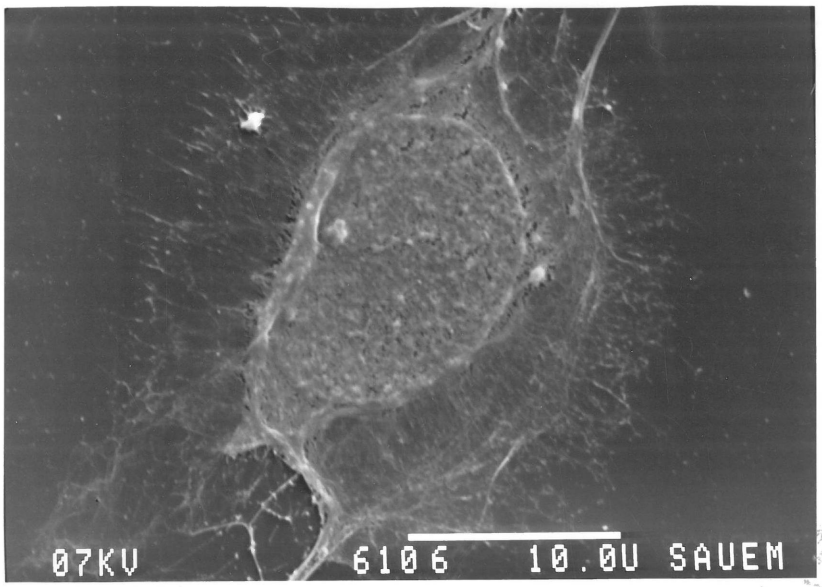


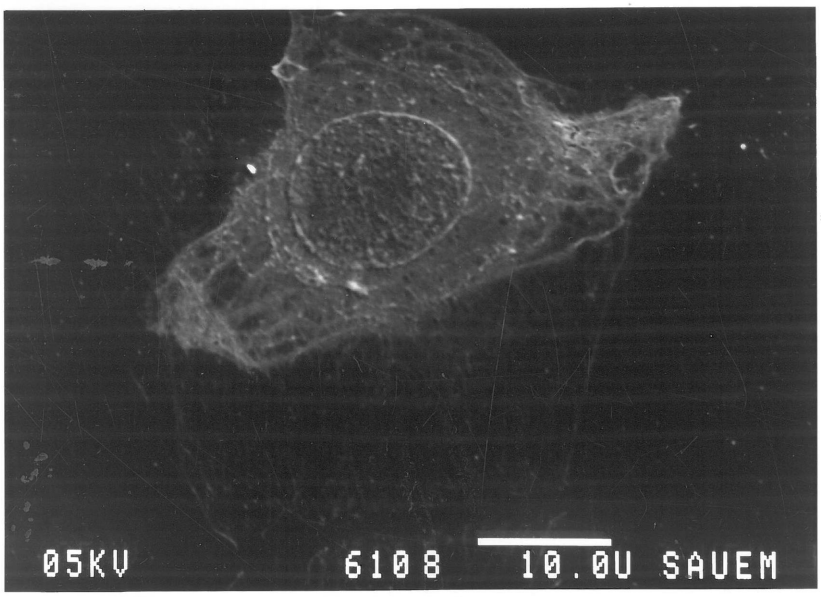
Plate 6

Micrographs of nucleoids of cells set in PBS agar and stained with ethidium bromide.

(a) Lysed in SDS pH 9.5

(b) Lysed in NLS pH 9.5


07KV
6106
10.0U SAUEM


05KV
6108
10.0U SAUEM

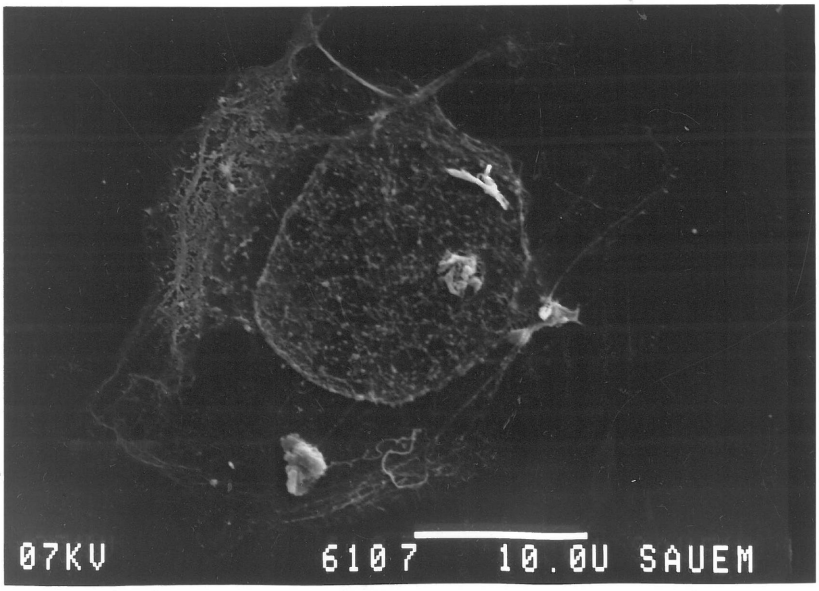

07KV
6107
10.0U SAUEM

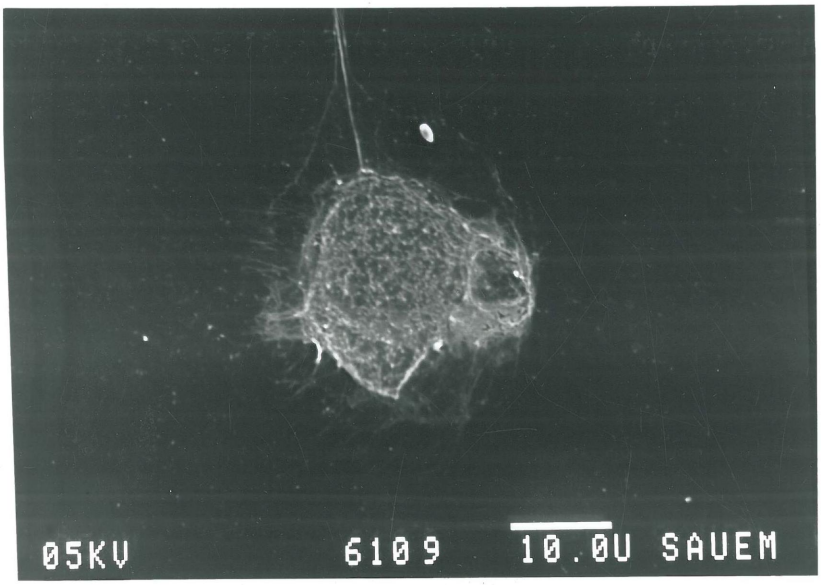
Plate 8

Scanning electron micrograph of nucleoids of cells lysed in triton and 1M NaCl while attached to the culture flask surface. Bars give scale in microns.

(a)

(b)

(c)


05KV
6109
10.0U SAUEM

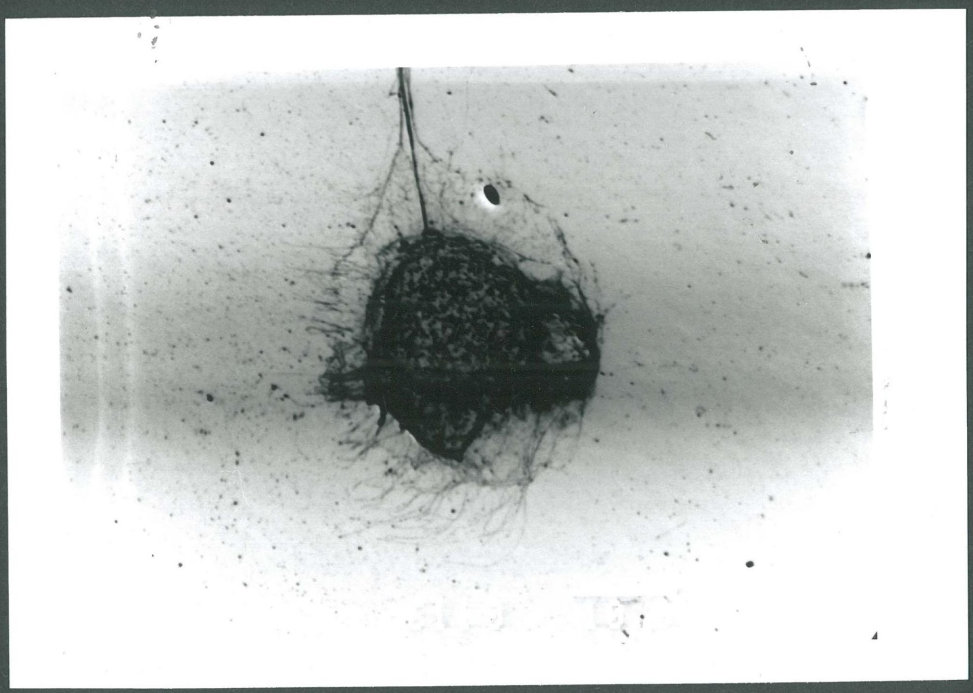
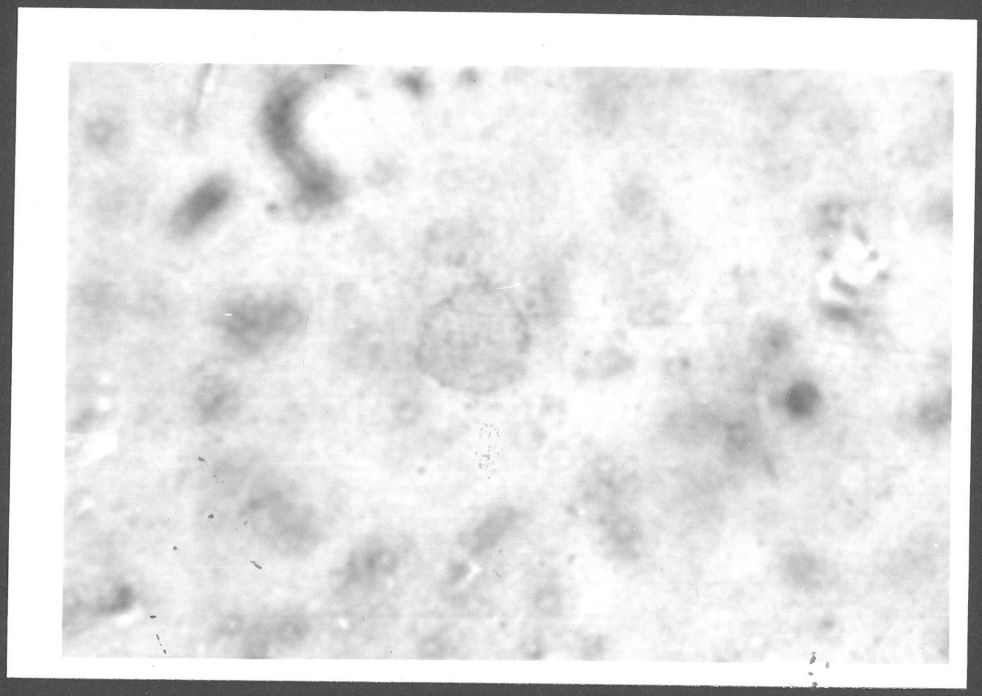


Plate 9

Scanning electron micrograph of a nucleoid of a cell lysed in triton and 1M NaCl while attached to the culture flask surface. Bars give scale in microns.

(a)Photographed as seen

(b) Photographed in reverse



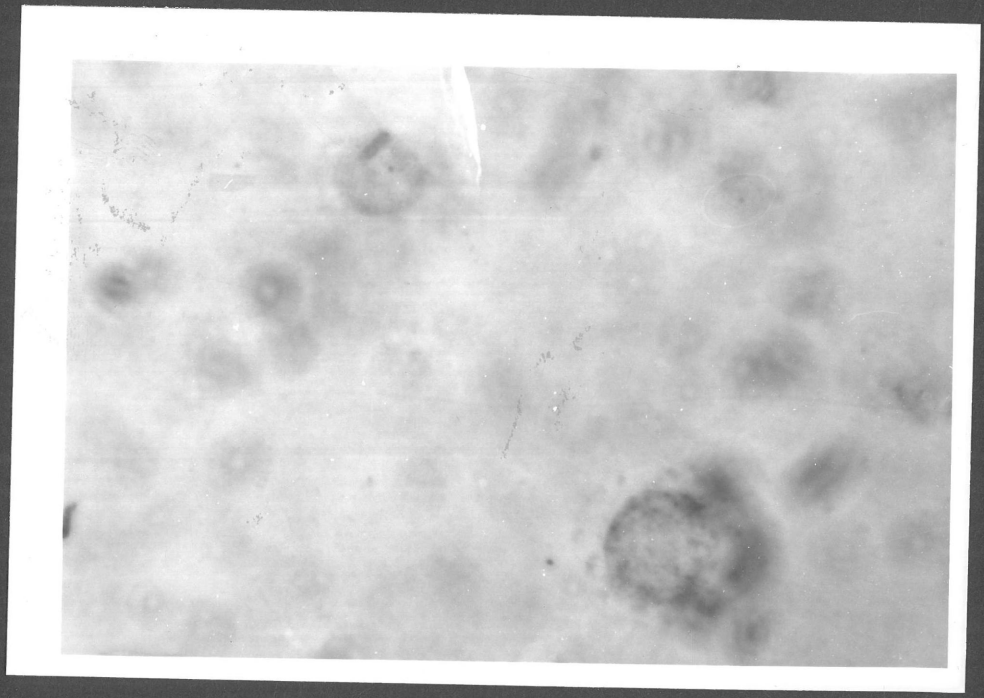
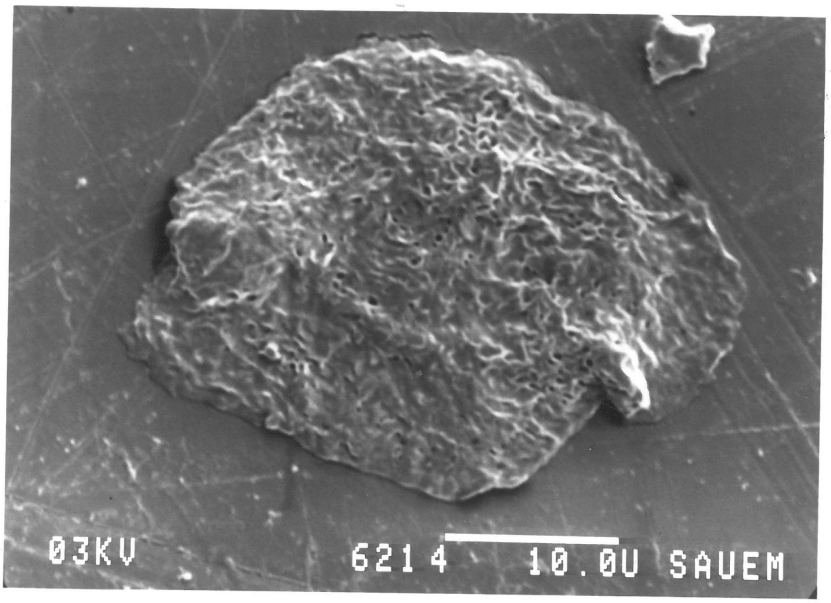


Plate 10

Micrographs of residues seen after cells were set in DW agarose, lysed in triton and 1M NaCl, treated with DNase and stained with Giemsa.

(a) Centre diameter 19.2 μm and 15.6 μm.

(b) Centre diameter 18 μm


03KV
6214
10.0U SAUEM

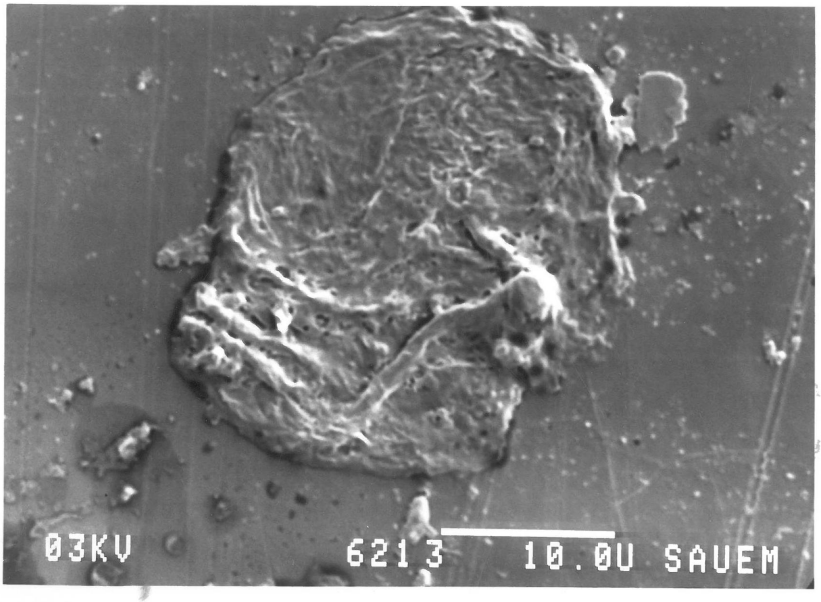

03KV
6213
10.0U SAUEM

Plate 11

Scanning electron micrographs of residues seen after cells were lysed while attached to the culture flask surface then treated with DNase. Bars show scale in microns.

(a)

(b)

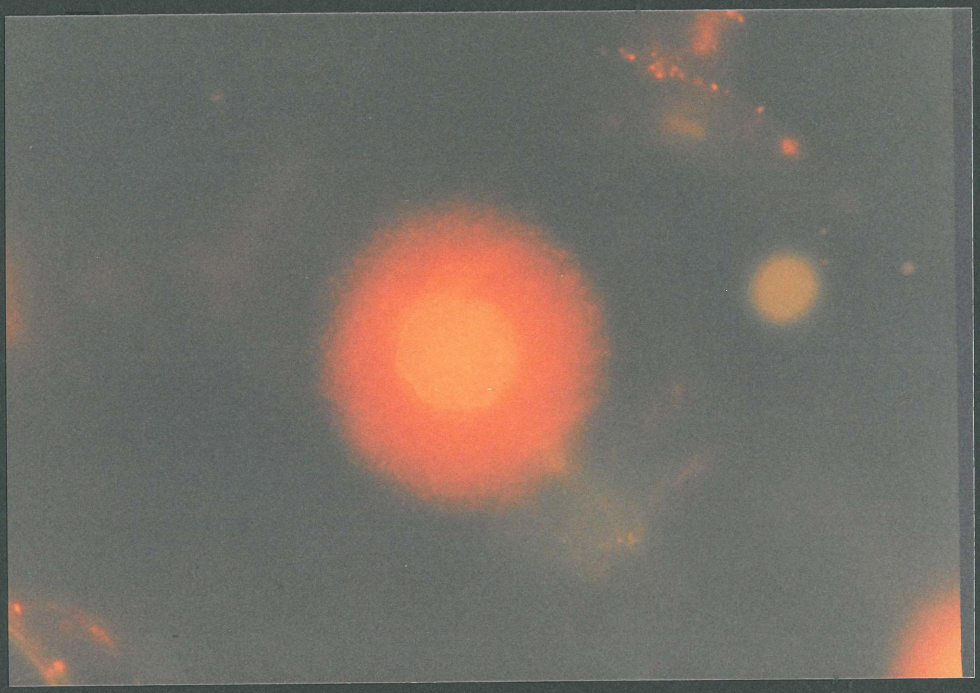
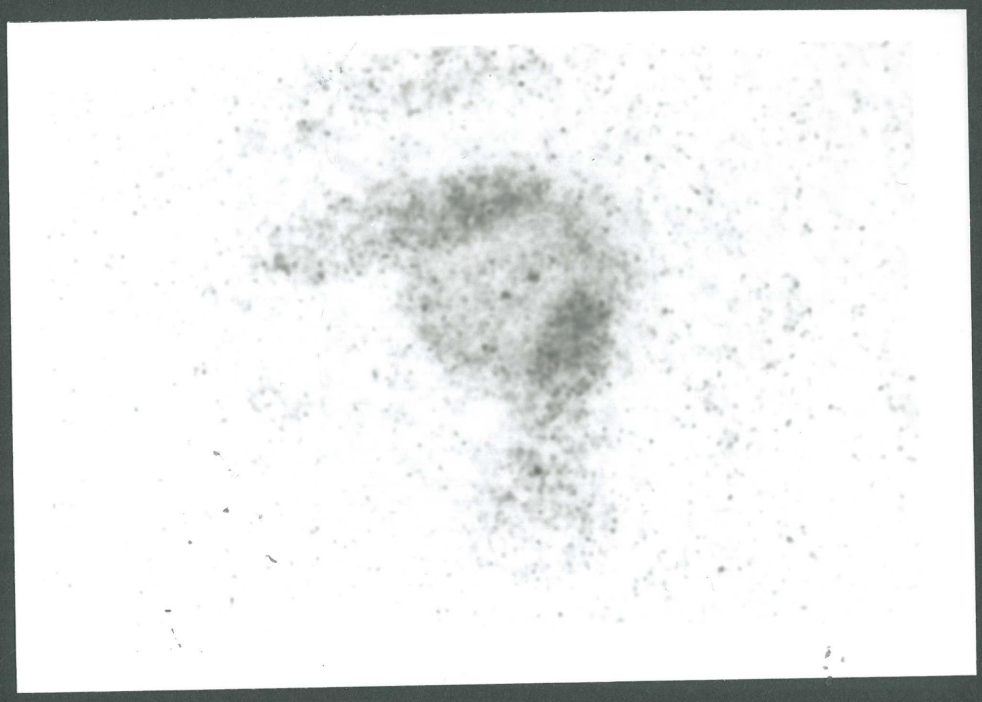


Plate 12

Micrographs of nucleoids treated with rabbit polyclonal 67 kD lamin protein antibody and stained with FITC-labelled anti-rabbit. The range of morphologies seen are demonstrated.

(a)

(b)



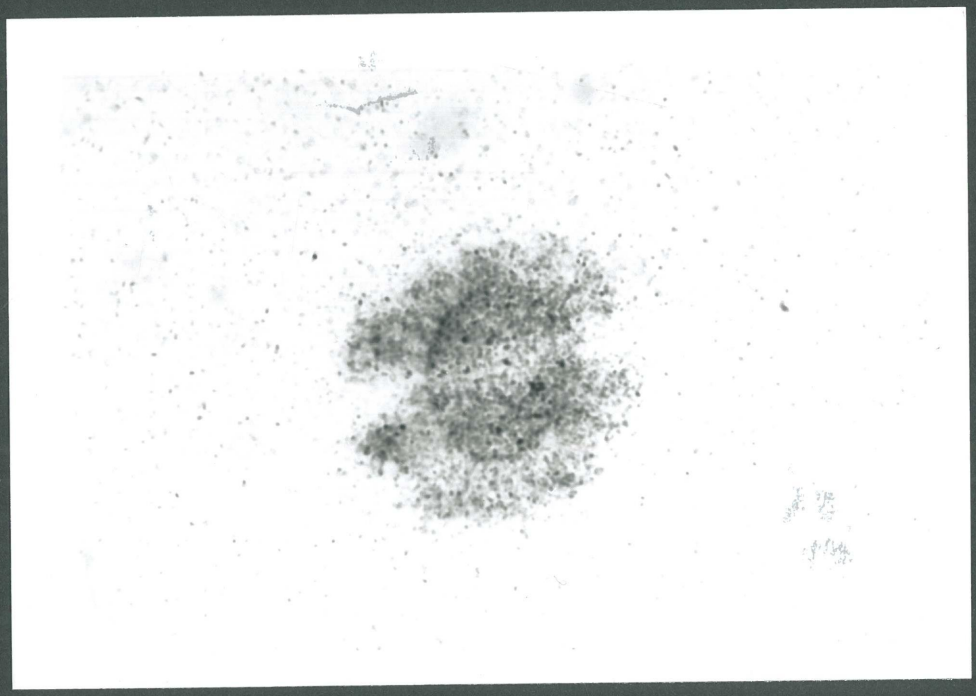
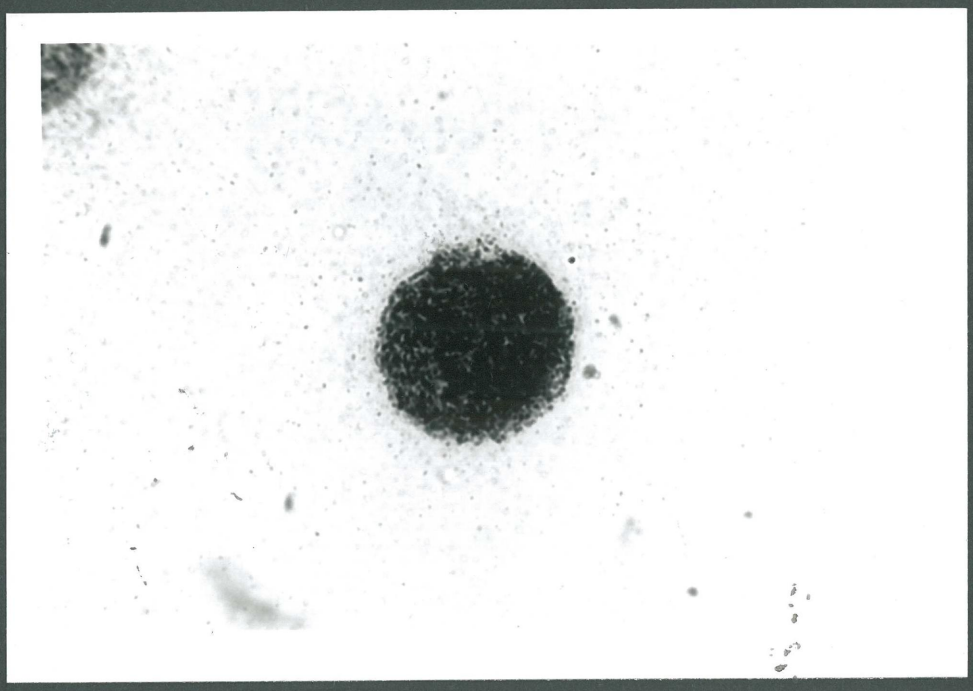


Plate 13

Micrographs of nucleoids prepared one hour after electroporating cells. Illustration of the nucleoid morphologies seen where 'bands' of damage occur across the structure.

(a)

(b)



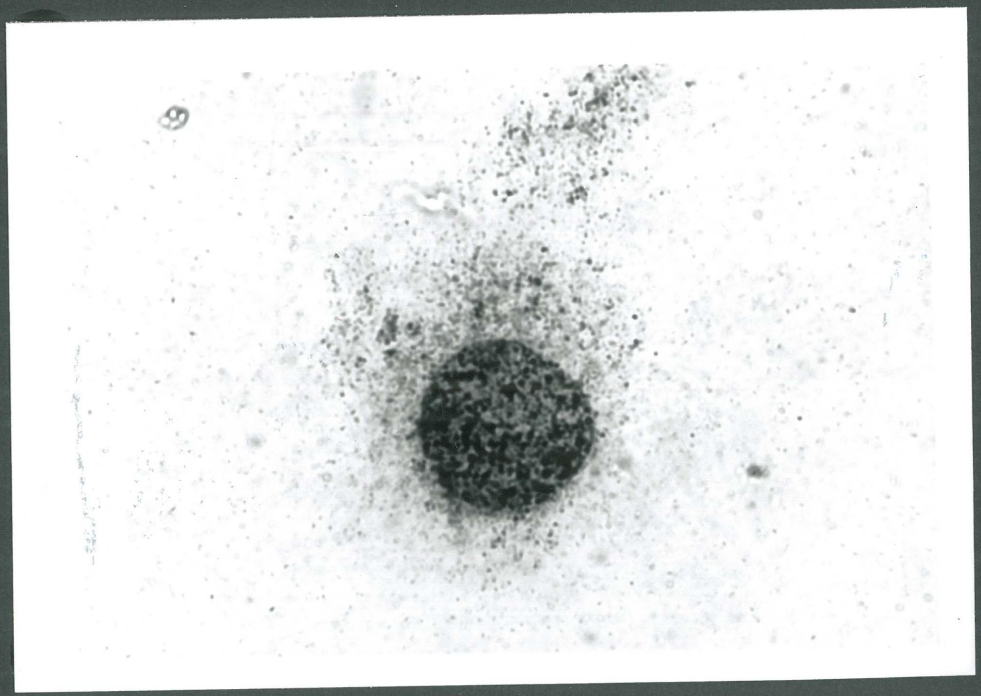
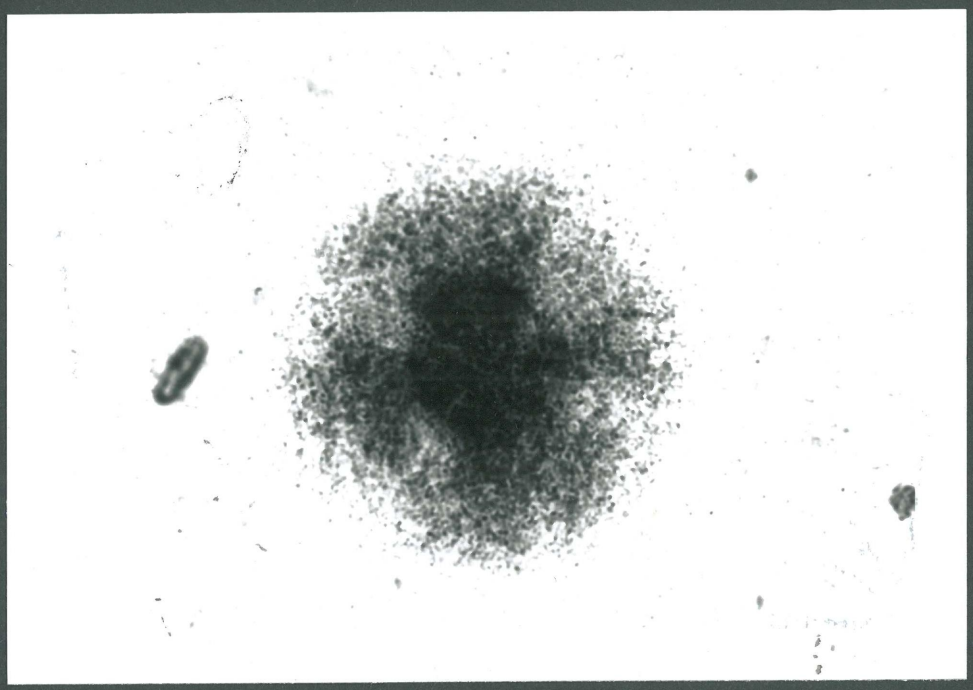


Plate 14

Micrographs of nucleoids prepared one hour after electroporating cells. Illustration of the nucleoid morphologies seen where the central region remains intact but much of the outer 'halo' region is missing or greatly altered.

(a)

(b)



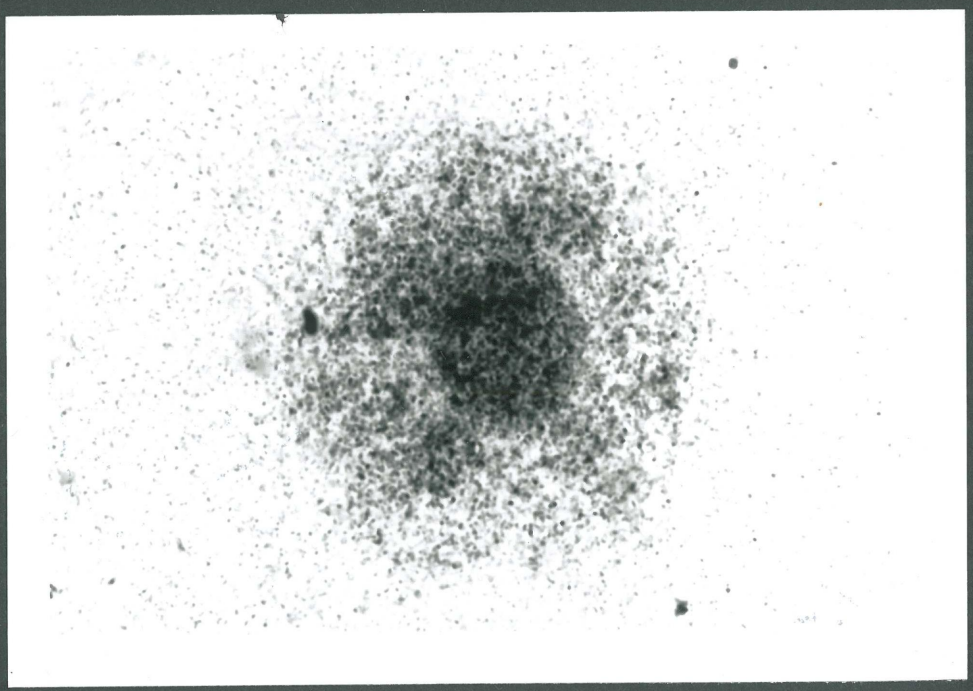
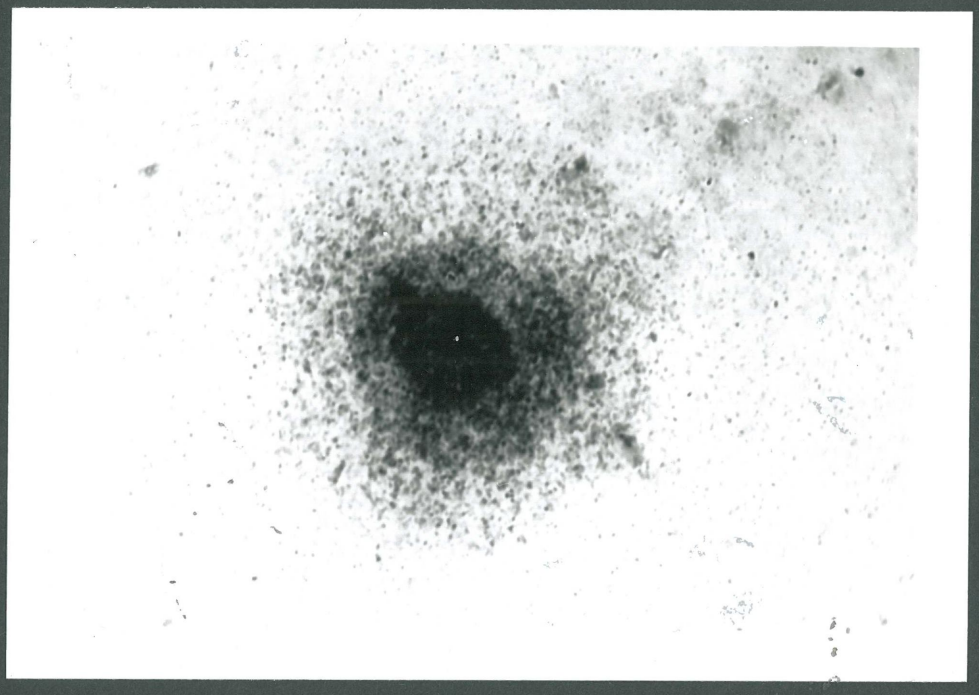


Plate 15

Micrographs of nucleoids prepared immediately after trypsinizing cells for 15 minutes.

(a)

(b)



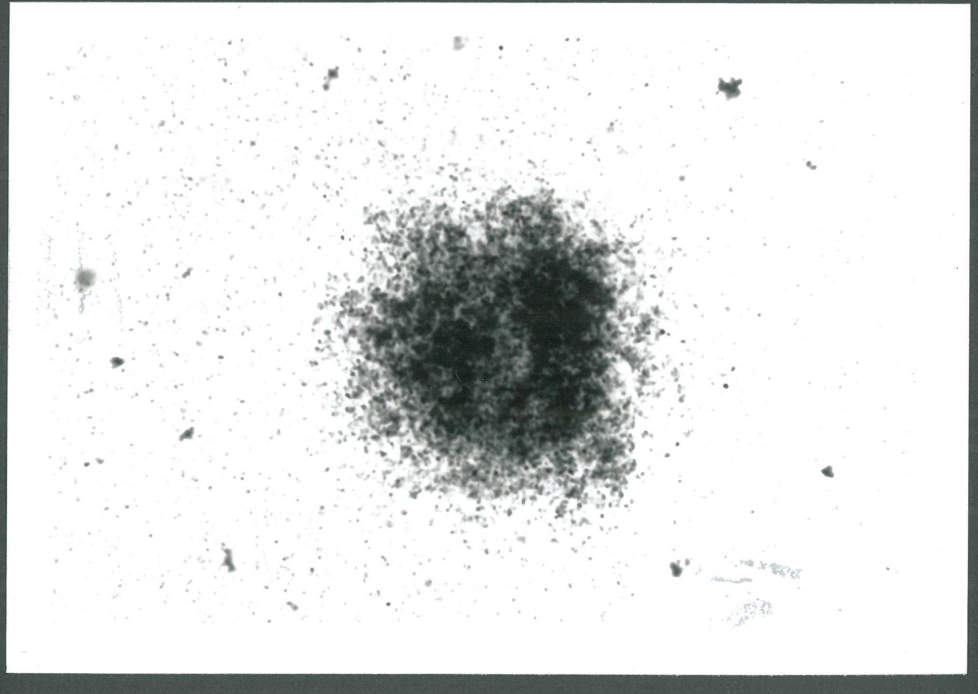


Plate 16

Micrographs of nucleoids prepared immediately after trypsinizing cells for 15 minutes.

(a)

(b)

APPENDIX III

Accepted for publication in

MUTATION RESEARCH

9th October 1989

EFFECTS OF TRYPSIN ON X-RAY INDUCED CELL KILLING, CHROMOSOME ABNORMALITIES AND KINETICS OF DNA REPAIR IN MAMMALIAN CELLS

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St Andrews, Fife KY16 9TS, UK.

Text pages: 13
Figures: 7

Summary

When cells are trypsinized before irradiation a potentiation of X-ray damage may occur. This is known as the 'trypsin effect'. Potentiation of X-ray damage on cell killing was seen in V-79 Chinese hamster cells but was marginal in Chinese Hamster Ovary (CHO K1) cells and not evident in murine Ehrlich ascites tumour (EAT) cells. Trypsinization did however increase the number of X-ray induced chromosomal abnormalities in all three lines.

To investigate the possibility that trypsin acts by digestion of proteins in chromatin, further experiments were performed to monitor DNA damage and repair. Induction of DNA breaks by X-rays was unaffected by trypsin but trypsinized EAT (suspension) cells repaired single strand breaks (ssb) less rapidly than controls indicating an inhibitory effect of trypsin on ssb repair. However double strand break (dsb) repair was unaffected by trypsin. It was also found that the EDTA solution in which the trypsin was dissolved also contributes to the inhibition of dsb repair.

The results show that trypsinization can enhance X-ray induced cell killing, chromosomal damage and DNA repair, the effect varying between cell lines.

Introduction

Trypsinization of cultured cells is employed in virtually all assays of cellular, cytogenetic and genetic damage resulting from exposure of cells to radiation or other genotoxic agents. Radiation damage can be mimicked by treatment of permeabilized cells with restriction endonucleases (RE) (Bryant, 1984; 1985) and trypsinization is in some assays used prior to treatment with RE (Obe and Winkel, 1985) - this procedure may assist entry of the RE but may also result in the entry of trypsin into cells. If the trypsin effect were due to the interaction

of trypsin with intracellular proteins *eg.* the histones or non-histone proteins associated with DNA, this could potentiate the effect of the RE by uncovering restriction sites. In order to investigate the underlying mechanisms of the trypsin effect we have examined the influence of trypsinization on X-ray induced cytogenetic and DNA damage, and on the capacity of cells to repair DNA damage.

The action of trypsin alone on cells has been widely investigated. Rinaldini (1959) for example found no evidence of cell killing due to routine trypsinization, although it is known to be toxic at high concentrations (Rous and Jones, 1916) and has since been shown to affect various cell properties (Hodges *et al*, 1973; Puck and Marcus, 1955; Hebb and Chu, 1960) including the potentiation of X-ray cell killing (Barendsen and Walter, 1964; Phillips and Tolmach, 1964; Berry *et al*, 1966; Lehnert, 1975; Bryant and Parker, 1979; Raaphorst *et al*, 1979; Djordjevic, 1979) - this is known as the 'trypsin effect'. The effect is generally found to revert to normal within 24 hours (Barendsen and Walter, 1964).

Trypsin is thought to detach cells by removing part of the cell coat involved in cell adhesion (Cook *et al*, 1960; Uhlenbruck, 1961; Laws and Strickland, 1961). The cell surface components are left behind on the flask (Weiss and Coombs, 1963) and appear to make it easier for cells to reattach to the same flask (Weiss, 1961), while the trypsin stays attached to the cell, remaining active (Poste, 1971) and preventing the short-term reformation of the glycoprotein cell coat material. The trypsin is gradually lost from the surface over the following 24 hours.

Lamb and Ogden (1987) postulated that trypsinization caused the cells to become more permeable due to the detachment of 'feet' holding the cells onto the substrate. Hodges *et al* (1973) showed that when cells are trypsinized the enzyme is not only bound to the cell surface but is also taken up into the cell; the presence of the enzyme in the

cytoplasm, nucleus and nucleolus was seen to persist for up to 48 hours after trypsinization. It has not been proven however whether trypsin is still active within the cell and so it is not known whether it could be acting on essential components within the cell as well as on the cell membrane.

Trypsin has a very specific action, only cleaving amino acids at lysine and arginine (ie basic) residues and so would not attack DNA *per se* but could act on other components in chromatin. Histones have a high proportion of basic amino acids and so could be a target for trypsin, once it enters a cell. Alternatively, trypsin could act on important (*eg* repair) enzymes in the cell, impairing their function.

The trypsin effect has been shown to occur in several cell lines by clonogenic assay. As the time between trypsinization and irradiation increases, the radiosensitivity, while decreasing overall, fluctuates in a cyclic, repeatable way which is thought to be due to trypsin causing partial cell synchrony (Barendsen and Walter, 1964; Berry *et al*, 1966; Lehnert, 1975; Raaphorst *et al*, 1979; Phillips and Tolmach, 1964) as the radiosensitivity of cultured mammalian cells fluctuates throughout the cell cycle (Sinclair and Morton, 1961; 1964; Belli and Shelton, 1969). Phillips and Tolmach (1964) and Berry *et al* (1966) thought that accumulation of cells in a more resistant phase of the cell cycle could also be important. Sun *et al* (1986) suggested that trypsin could interact with radiation damage to the cell membrane, while Reddy *et al* (1989) postulated that the trypsin effect was mainly due to morphological changes of the cell on trypsinization, spread cells being better able to repair damage than those in suspension.

Here we report experiments using several strategies in an attempt to gain information on the mechanism of the trypsin effect. Firstly we have investigated the effect of trypsin on three cell lines. Secondly we have investigated whether trypsin affects radiation-induced cytogenetic

damage. Thirdly we have sought evidence for chromatin as a sensitive target for trypsin within the cell by using two methods of measuring strand breakage: DNA unwinding and neutral elution.

Materials and Methods

Cell culture

Two Chinese hamster cell lines were used: V-79 and CHO K1; both of which were routinely cultured in 75cm^2 tissue culture flasks (Sterilin) in Eagle's minimal essential medium (MEM) supplemented with 15% foetal calf serum (FCS) and non-essential amino acids. Cells were trypsinized from flask surfaces using 2 washes with a standard trypsin/EDTA solution: 0.05% trypsin (Bacto Trypsin Difco), 0.2g/l EDTA in buffer/salts solution (8g/l NaCl, 0.2g/l KCl, 1.15g/l Na_2HPO_4, 0.2g/l KH_2PO_4), incubated at 37^{0}C for 10 minutes then resuspended in MEM. Non-trypsinized cells were scraped off the flask surface with the sterile rubber end of a 1ml syringe plunger.

Murine Ehrlich ascites tumour (EAT) cells were also used. These were grown in suspension and passaged daily in A2 medium (Illiakis and Pohlit 1979). EAT cells were used so that non-trypsinized controls could be obtained without the need for scraping cells off dishes. For trypsin or buffer treatment of EAT cells, samples of cells were dispensed into 10ml sterile 'V' centrifuge tubes and pelleted (5 minutes at 1000rpm). The medium was aspirated and the pellet loosened by vortexing, then 5ml of either trypsin or buffer (the solution in which trypsin is dissolved) was added to each tube and incubated for 5, 10, or 15 minutes at 37^{0}C. For the last 3 minutes the cells were centrifuged at room temperature so that exactly at the end of the incubation time the trypsin could be drawn off and the cells resuspended in fresh growth medium.

Assays for clonogenic survival

Clonogenic assays for V79 and CHO K1 cells were performed using standard procedures. After treatment and appropriate dilution, cells were plated in 6cm plastic dishes with 5ml MEM and incubated for 6 days. After fixation, colonies were stained in Giemsa.

EAT cells were assayed in soft agar as previously described (Iliakis and Pohlit, 1979).

Assays for chromosome abnormalities

Anaphase aberrations

Anaphase abnormalities (fragments and bridges) were assayed in EAT cells because the presence of many small telomeric chromosomes made metaphase scoring unreliable. The technique used was as described previously (Bryant 1983). Before irradiation cells were treated with trypsin or buffer for 10 minutes. 400 anaphases were scored per sample for abnormalities; 1000 mitoses were scored per sample to determine the mitotic index.

Metaphase aberrations

Metaphase aberrations were assayed in CHO and V-79 cells. Cells in $25cm^3$ flasks were trypsinized (6 minutes) then fresh medium prewarmed to 37^0C was added. In other flasks the medium was changed without trypsinization. Some flasks were then irradiated with either 3 Gy or 4.2 Gy, after which all flasks were incubated for 16 or 20 hours at 37^0C (including the last hour in $4x10^{-4}$ug/ml colcemid) then harvested by standard procedures.

Assay of DNA strand breakage

The DNA unwinding assay

The DNA unwinding assay (Ahnstrom and Erixon, 1973) was performed essentially as described by Bryant and Blocher (1980). EAT

cells at a concentration of $3x10^5$ cells/ml were labelled with $2x10^3$ Bq/ml ^{3}H-thymidine (specific activity $1.5x10^{12}$ Bq/mmol); 5uMol/ml unlabelled thymidine was also added. Cells were then incubated for 40 hours to allow labelling then treated with trypsin or buffer as above.

After irradiation, DNA unwinding was carried out in 0.03 mol/l NaOH in 1mol/l NaCl for 1 hour. For repair experiments, cell samples were X-irradiated with 12 or 50 Gy and incubated at 37^0C for time intervals between 0 and 60 minutes (short term repair) or 2-8 hours (long term repair).

Neutral Filter Elution

The non-unwinding DNA filter elution technique was used as developed by Bradley and Kohn (1979), with minor modifications (Okayasu and Iliakis, 1988). Exponentially growing EAT cells were diluted to $3x10^5$/ml and labelled for 40 hours with $4x10^3$ Bq/ml ^{3}H (specific activity $1.5x10^{12}$ Bq/mmol); 50umol/ml unlabelled thymidine was also added. Internal standards were not used. $5x10^5$ cells were loaded onto each filter; lysis was for 1 hour at 60^0C in a solution of 2% sodium-N-laurylsarcosine (NLS) and 0.5mg/ml proteinase K set to pH 7.4. After lysis, DNA was eluted from the filters with 40 ml tetrapropylammonium hydroxide set to pH 7.4 at approximately 2.8 ml/hour (15-16 hours). The amount of activity eluted from each filter as a fraction of the total activity in each sample (filter activity plus eluted activity) was used as a measure of the DNA damage to each sample. The background (unirradiated) fraction eluted (f_e) was subtracted from each f_e value initially calculated to give the values quoted here.

X-irradiation

Cells were exposed to X-rays (250kV, 14mA, 0.5mm Cu filter) from a Siemens therapy unit giving a dose rate of 0.75 Gy/min or 5.8 Gy/min and were contained either in $25cm^3$ plastic flasks or 7ml plastic bijou

bottles. Doses were checked by a ferrous sulphate method (Frankenberg, 1969).

Results

Survival curve experiments

The survival of V-79 cells after X-irradiation at different times after trypsinization is shown in figure 1a. After 12 Gy, the surviving fraction fluctuated with time between trypsin and irradiation, showing an overall increase or recovery which was also seen after 6 Gy, an effect previously demonstrated for CHO cells (Berry *et al*, 1966). This effect was not observed over shorter incubation times of up to 1 hour between trypsinization and X-irradiation (Figure 1b).

A trypsin effect was not observed for V-79 cells trypsinized 24 hours before irradiation or scraped 45 minutes before irradiation (Figure 2a); cells trypsinized 90 minutes before irradiation showed the largest trypsin effect, lower survival (twofold) with a change in the shoulder was evident (Figure 2a). The plating efficiencies of trypsinized V-79 cells were between 50 and 60 per cent but was only 8 per cent for scraped cells. Much less potentiation of X-ray killing was seen for CHO cells (Figure 2b) and none was apparent for EAT cells (Figure 2c).

It was decided that a realistic time interval between trypsinization and irradiation (to include cell treatment as required in a typical experiment) was 45 minutes, so this was used in the following experiments.

Chromosomal aberrations

Anaphase aberrations in EAT cells

Stationary cells were incubated in fresh medium following trypsin or buffer treatment and X-irradiation (4 Gy). After some 15-18 hours they entered cycle, the subsequent wave of mitoses peaking at 24-26

hours (Figure 3a). The percentage of normal anaphases in these cells increased slightly through the 21-28 hours incubation from 24.9% to 30.7%. When using unirradiated cells, 72% normal anaphases (0.4 aberrations per cell) were found in the trypsin treated samples and 69% normal anaphases (0.43 aberrations per cell) in those buffer treated. When trypsin treated cells were X-irradiated with a dose of 4 Gy a decrease was seen in the percentage of normal anaphases throughout the 21-28 hours' incubation (results not shown) and an increase in the average number of abnormalities per cell (Figure 3b) compared with buffer treated samples.

In addition, the average number of aberrations per cell in the trypsin treated cells increased with time of incubation of the cells in fresh medium; this trend was not so significant in the buffer treated cells.

The mitotic index was too low at longer time intervals for there to be enough anaphases to score.

Metaphase aberrations in CHO cells

In both the V-79 and CHO cell lines a larger number of aberrations per 100 cells was seen for cells trypsinized and X-irradiated than the sum of the number of aberrations for cells just trypsinized or X-irradiated (Figure 4).

DNA strand breaks

DNA unwinding experiments with V-79 cells

No difference was seen between the unwinding kinetics of trypsinized and scraped cells (results not shown), or between the dose-effect curves of trypsinized and scraped cells up to an X-ray dose of 12 Gy - thought to represent the induction of DNA ssb (Figure 5a).

The repair kinetics of trypsinized and scraped V-79 cells after 12 Gy are shown in Figure 6a - thought to represent the repair of ssb.

The repair kinetics of the trypsinized cells were similar to those published for cells grown in suspension (Bryant *et al*, 1984) but the rate of repair in scraped cells was much slower. A possible reason for this emerged from the survival curve experiments. The plating efficiency of scraped cells was 8 per cent which is very low, indicating that most cells are killed by the scraping process. However, the survival curve indicated that those cells which were not initially killed were less sensitive to X-rays than those which were trypsinized. These surviving cells were only a small fraction of the total. When studying the repair kinetics, however, all cells scraped off the plates are included - both those few for which reproductive integrity is maintained and the majority which are damaged (and thus less able to repair) and will subsequently die.

It may be, therefore, that cells do not repair efficiently after trypsinization but V-79 cells scraped in this way are not suitable controls. It was decided, therefore, to carry out DNA unwinding experiments using suspension EAT cells obviating the need for scraping.

DNA damage and repair of EAT cells in suspension

As found for V-79 cells, no difference was seen in the unwinding kinetics of trypsinized and buffer-treated cells after 12 Gy (results not shown) or in the DNA unwinding dose-effect curves representing the induction of ssb between trypsin treated and control samples (Figure 5b).

The ssb repair kinetics of X-irradiated (12 Gy) EAT cells was similar in untreated and 15 minute buffer treated cells (figure 6b). 15 minute trypsinization however decreased the rate of ssb repair. Less of a decrease in ssb repair capacity was observed after shorter trypsinization times. No difference in dsb repair kinetics between trypsin and buffer treated cells was seen after 50 Gy (Figure 6c).

Neutral elution

No difference in the dose-effect curves for induction of damage (dsb) was observed, but these both showed a steeper response than completely untreated cells (Figure 7a). This result was unexpected since neither trypsin nor buffer treatment altered the DNA unwinding dose-effect curve.

The dsb repair kinetics (Figure 7b and c) after 40 Gy showed no significant difference between the repair in trypsin and EDTA/buffer treated cells, confirming the DNA unwinding results.

Discussion

The results show that pre-irradiation trypsin treatment caused a small increase (potentiation) of X-ray cell killing in V-79 cells (Figure 2a). The effect in V-79 cells represented essentially a change in the shoulder of the survival curve and a decrease in surviving fraction by a factor of about 2 but only a very marginal effect in CHO cells (Figure 2b) and no effect in EAT cells (Figure 2c). The differences in response of the three cell lines suggests they may have different sensitivities of outer cell membranes to trypsin; Sun *et al* (1986) suggested that the trypsin effect was a membrane phenomena while Reddy *et al* (1989) proposed that different radiosensitivities of attached and rounded (trypsinized) cells was the cause.

To investigate the molecular mechanisms underlying the trypsin effect, we studied the chromosomal and DNA responses to the combined effects of trypsin and X-rays. There was a clear effect of trypsinization on the frequencies of X-ray induced chromosomal aberrations in all three cell lines (Figures 3 and 4). This effect was not reflected in the survival curve results for CHO and EAT cell lines. Trypsin and buffer (EDTA) affected induction of DNA dsb as measured by neutral elution, although it is conceivable that this is an effect on the ability of

damaged DNA to elute through filters (*eg* by a decrease in viscosity) rather than an actual increase in breakage of DNA. Results obtained with the unwinding method indicated no difference in ssb induction (Figure 5).

The inhibition of ssb repair after trypsinization (figure 6) may support the observation of Kaufmann and Briley (1987) who showed that excision repair of trypsinized cells is 30% less efficient than for monolayers. Dsb repair was not inhibited by trypsin any more than by EDTA buffer (Figure 6 and 7). The theory of Reddy *et al* (1989) that the trypsin effect is due to change in cell morphology would only hold for clonogenic assay as the assays for chromosomal aberrations and DNA repair here showed a trypsin effect for EAT (suspension) cells. Reddy *et al* (1989) postulated that trypsinization may induce some chromosome contraction as induced by hypertonic salt or mitosis (Dettor *et al*, 1972). The rate and extent of DNA repair might be influenced by DNA accessibility (Wheeler and Wierowski, 1983). However a difference in DNA unwinding kinetics which would reflect any change in DNA conformation was not observed. Alternatively the trypsin effect could be caused indirectly by damage to the cell membrane leading to alterations in ionic balance or loss of vital molecules as suggested by Sun *et al* (1986).

In conclusion, we have verified the trypsin effect as previously reported and in addition shown that effects of trypsin on chromosomal damage in X-irradiated cells may be even more marked than those at the cellular level. Possibly linked to the cellular and chromosomal effects are the effects of trypsin and EDTA on the rate of repair of both ssb and dsb.

Acknowledgement

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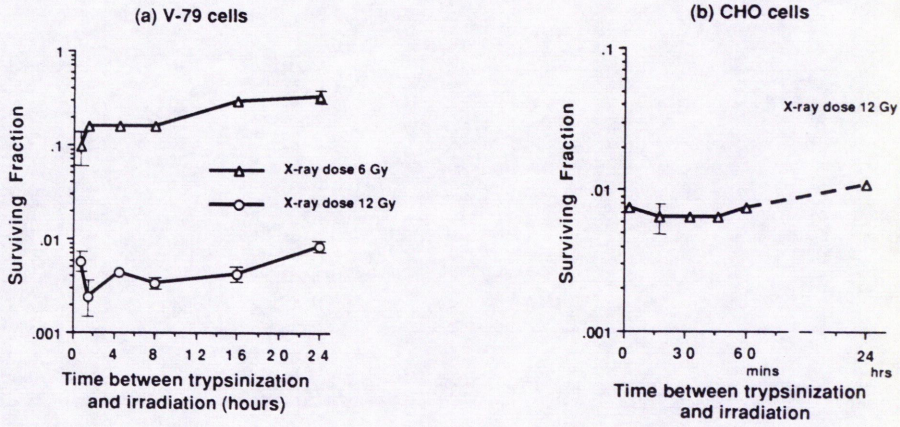

Fig. 1. Change in cell survival due to X-irradiation at different times after trypsinization. Multiplicity is corrected for where necessary. Vertical bars show SEM; where not shown are within symbol size.

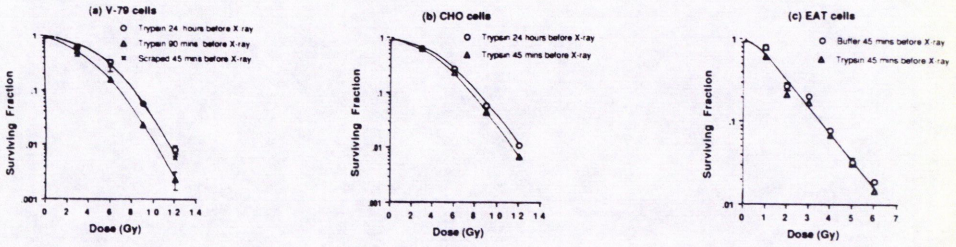

Fig. 2. Survival Curves. All trypsin and buffer treatment times: 10 minutes. Multiplicity is corrected for where necessary. Vertical bars show SEM; where not shown are within symbol size.

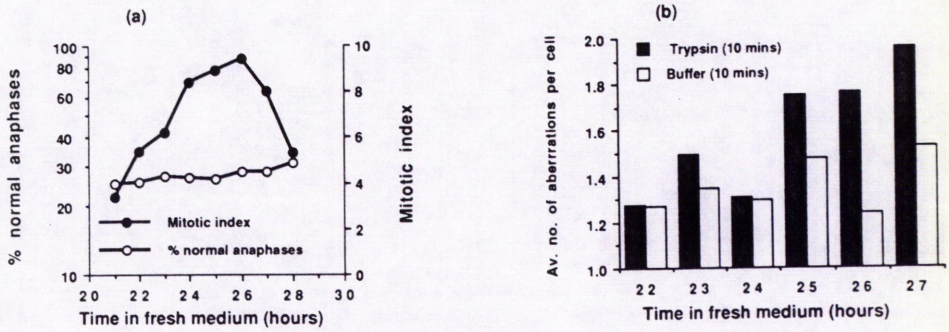

Fig. 3. Anaphase abnormalities in EAT cells after X-ray doses of 4Gy.

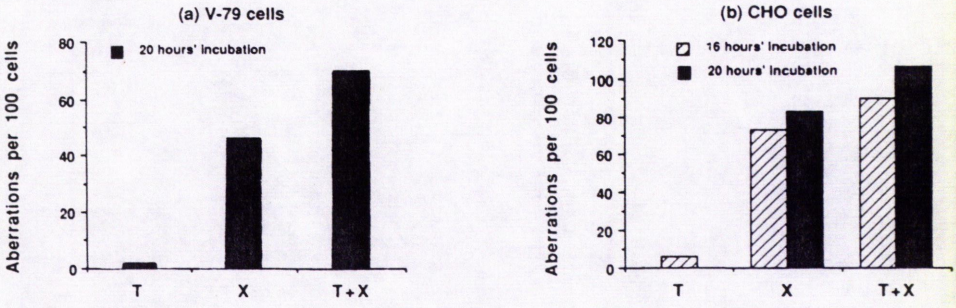

Fig. 4. Metaphase abnormalities in V-79 and CHO cells.
T=trypsinized, (a) X=X-ray dose 4.2 Gy; (b) X=X-ray dose 3 Gy.

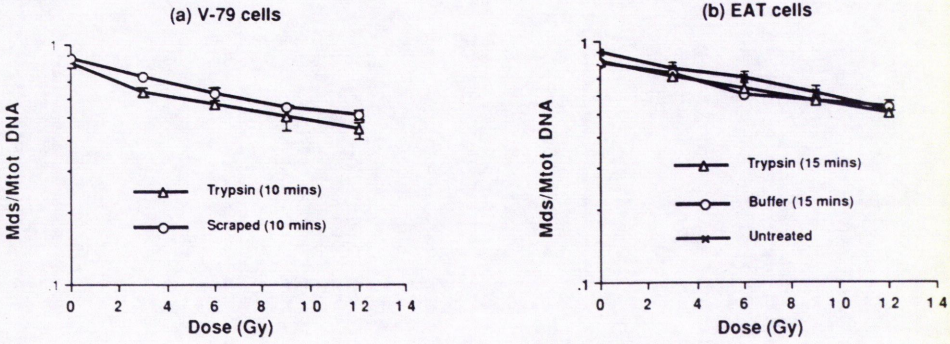

Fig. 5. DNA unwinding dose-effect curves. Vertical bars show SEM; where not shown, bar is smaller than symbol. Data is obtained as the proportion of the DNA that is double standed (Mds/Mtot DNA), thought to be proportional to the number of breaks present in the DNA.

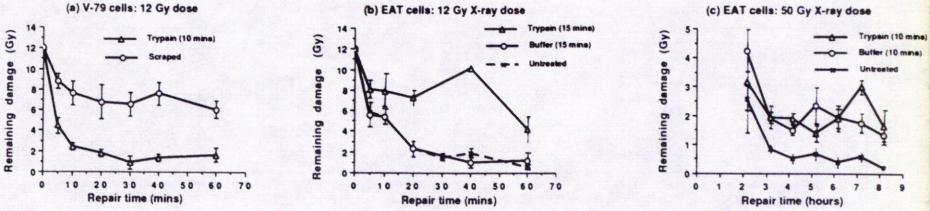

Fig. 6. DNA unwinding repair kinetics. Vertical bars show SEM. The original Mds/Mtot DNA data was converted to remaining damage (Gy) by reading the values off the dose effect curve.

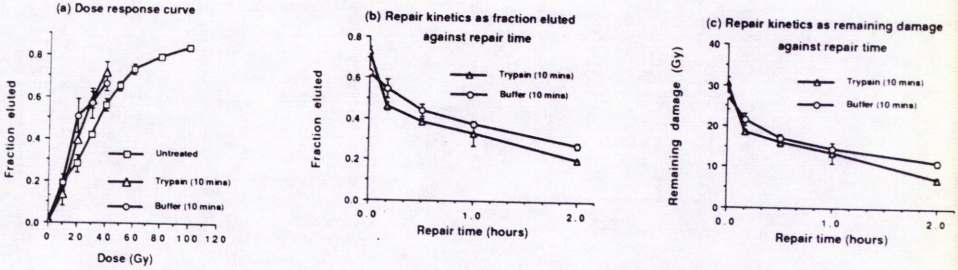

Fig. 7. Neutral elution dose-effect curve and repair kinetics. Vertical bars show SEM. The fe data was converted to remaining damage (Gy) by reading the values off the dose effect curve.